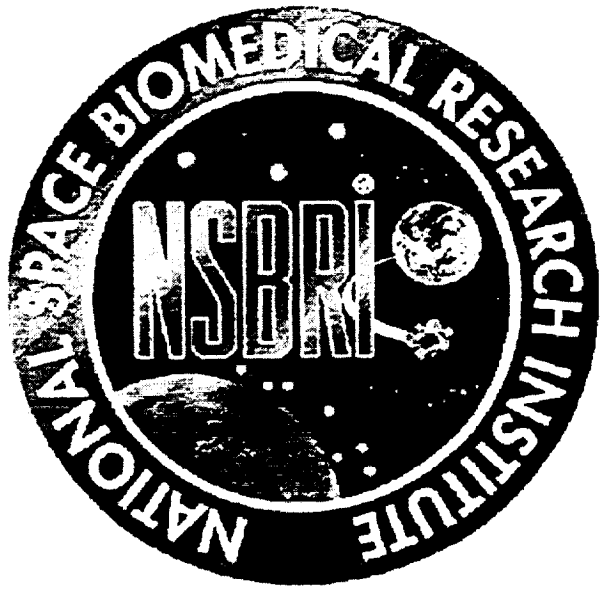
NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Annual Report

October 1, 1999 – September 30, 2000



Cooperative Agreement NCC 9-58

with the

National Aeronautics and Space Administration
Lyndon B. Johnson Space Center
Houston, Texas

September 30, 2000

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1.0 INTRODUCTION

This report summarizes the activities of the National Space Biomedical Research Institute (NSBRI) during FY 2000, the third full year of existence of the NSBRI's research program, and is prepared in accordance with Cooperative Agreement NCC 9-58 between NASA's Lyndon B. Johnson Space Center and Baylor College of Medicine (NSBRI).

2.0 BACKGROUND

The NSBRI is responsible for the development of countermeasures against the deleterious effects of long-duration space flight and performs fundamental and applied space biomedical research directed towards this specific goal. Its mission is to lead a world-class, national effort in integrated, critical path space biomedical research that supports NASA's Human Exploration and Development of Space (HEDS) Strategic Plan by focusing on the enabling of long-term human presence in, development of, and exploration of space. This is accomplished by:

- designing, testing and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight;
- defining the molecular, cellular, organ-level, integrated responses and mechanistic relationships that ultimately determine these impediments, where such activity fosters the development of novel countermeasures;
- establishing biomedical support technologies to maximize human performance in space, reduce biomedical hazards to an acceptable level, and deliver quality medical care;
- transferring and disseminating the biomedical advances in knowledge and technology acquired through living and working in space to the general benefit of mankind, including the treatment of patients suffering from gravity- and radiation-related conditions on Earth; and
- ensuring open involvement of the scientific community, industry and the public at large in the Institute's activities and fostering a robust collaboration with NASA, particularly through NASA's Lyndon B. Johnson Space Center.

The NSBRI was established in April 1997 following competitive selection by NASA. Primary support for the NSBRI's activities is furnished by NASA through a cooperative agreement although funds to support Institute activities also come from several sources, including the institutions involved in carrying out the NSBRI's programs. The cooperative agreement award is for a five and one-half year base period, lasting until September 30, 2002, and three five-year optional extensions. Base funding from NASA for FY 1998 and FY 1999 was approximately $10 million annually. Base funding from NASA for FY 2000 was increased to approximately $14 million to to develop the infrastructure to support planned program growth anticipated to begin in FY 2001.

The NSBRI is governed by a consortium of twelve institutions that includes Baylor College of Medicine, Brookhaven National Laboratory, Harvard Medical School, The Johns Hopkins University School of Medicine and the Applied Physics Laboratory, Massachusetts Institute of Technology, Morehouse School of Medicine, Mount Sinai School of Medicine, Rice University, Texas A&M University, the University of Arkansas for Medical Sciences, the University of Pennsylvania Health System, and the University of Washington. The Institute's headquarters are located in Houston at Baylor College of Medicine.

The initial Institute research program consisted of eight research teams carrying out 37 three-year projects and four special one-year "synergy" projects designed to bridge between discipline research team activities and create an appropriate atmosphere for future interdisciplinary research. Because of the nature of the competitive process used by NASA to select the NSBRI, most of the initial program has been carried out at the seven original consortium institutions. There are, however, no restrictions concerning institutional participation in Institute activity. During the third and final year of the initial program, 135 investigators at 31 institutions and government laboratories (see Appendix A) worked on NSBRI projects.

The management plan for the Institute is based on the model used by the National Institutes of Health. An independent Board of Scientific Counselors is responsible for assuring excellence in the Institute's intramural program through independent external peer review, and an External Advisory Council is responsible for advising Institute management concerning programmatic effectiveness. The NSBRI also has a User Panel of former and current astronauts and flight surgeons responsible for assuring that the research program is focused squarely on astronaut health and safety. An Industry Forum of representatives of space and biomedically-related industries assists the Institute in developing industry participation in NSBRI and in timely technology transfer. In addition to its research program, the NSBRI has developed a vital education and outreach program which takes advantage of the Institute's core research activities.

3.0 RESEARCH PLAN

As described in the original proposal to establish the NSBRI, the Institute's initial strategic research agenda involves eight teams of scientists focused on:

- *Bone Loss* – Addressing the loss and weakening of bone during space flight with the inherent fracture risks;
- *Cardiovascular Alterations* – Addressing inflight increase of cardiac dysrhythmias and postflight impairment of the cardiovascular response to orthostatic and exercise stress;
- *Human Performance* – Addressing maintenance of high cognitive performance and vigilance despite environmental stress and sleep disturbances;
- *Immunology, Infection and Hematology* – Addressing the potential for immune system impairment and altered susceptibility to infection, increased allergic response, decreased blood volume and postflight anemia;
- *Muscle Alterations and Atrophy* – Addressing the loss of skeletal muscle mass, strength and endurance that accompanies space flight;
- *Neurovestibular Adaptation* – Addressing the problems of space motion sickness and disorientation during flight and the postflight problems of balance and gaze disorders;
- *Radiation Effects* – Addressing the problem of increased cancer risk caused by the natural space radiation environment; and
- *Technology Development* – Developing instrumentation that will enhance the research of the other teams and transferring the technology to industry for the benefit of society.

Each research team consists of investigator groups working on complementary projects focused on a common theme. Team management and coordination is the responsibility of a program director called a Team Leader while overall scientific direction is the responsibility of the Institute Director and Associate Director. As mentioned above, the initial Institute research program, caried out from October 1, 1998 through September 30, 2000, involved 37 three-year projects and 4 one-year projects. For FY 2000, the average annual funding per project was approximately $242,000 (Direct + Indirect Costs). Appendix B provides a summary of each project, including funding information for FY 1998, 1999 and 2000. Further details concerning these projects are provided in Appendix C, containing the final reports for the initial research program, and on the NSBRI web site: www.nsbri.org.

In addition to this core intramural research program, the NSBRI has developed a joint program with the National Institute on Deafness and Other Communication Disorders (NIDCD) that jointly funds six competitively awarded extramural grants related to the dynamic adaptation of central vestibular function, an area of common interest. Appendix D provides funding information for this five-year joint program initiated in FY 1999; NIDCD is contributing over $1.1 M to this program in FY 00, while the NSBRI is contributing $210 K from private sources.

During FY 2000, the NSBRI continued to develop its relationship with the Russian Institute for Biomedical Problems (IBMP) in Moscow. Appendix E provides the agenda for a special countermeasure course taught by the research staff of the IBMP in Houston in January 2000. This course was made available to NSBRI and NASA researchers. The entire course was filmed and is available for future use. In addition, the agreement (included in Appendix E as well) that was signed to enable this course also included the preparation of special reports to the NSBRI on Russian countermeasures. These reports have been received and distributed to the NSBRI research teams.

Table 1 presents the summary schedule of major NSBRI activities taking place in FY 2000. The activities ranged from workshops and retreats to management-related meetings of the Council and Board designed to provide guidance and oversight to the Institute's programs.

The program and project summaries of Appendix B and the final reports of Appendix C contain the discoveries and findings of the first three years of the research program initiated in FY 1998. *(Note that the pre-publication results presented in these Appendices is intended for NASA internal use only as these results are privileged.)* A large number of results have been published and Appendix F provides a list of papers, reports, abstracts and presentations resulting from full or partial NSBRI support.

3.1 Research Announcements

During the second year of NSBRI's operations, after meetings with Mr. Goldin, the NASA Administrator, and other NASA officials (see the NSBRI Annual Report for FY 1999), a strategy to expand the Institute's research program was developed and agreed upon. During FY 2000, that strategy was implemented.

In December 1999, a research announcement focused on the development of four new research teams was released. This announcement is included in Appendix G. It was a product of four workshops involving the scientific community held in the summer of 1999. Similarly, in February 2000, a research announcement was released that focused on expansion of the program

Table 1. MAJOR NSBRI ACTIVITIES October 1, 1999 – September 30, 2000		
DATE	**ACTIVITY**	**LOCATION**
October 25 – December 8, 1999	8 Research Area WORKSHOPS – Original Research Areas	Houston
October 27	NSBRI Board Committee Reviews New Team Leader Candidates	Houston
November 17-18	New Team Leader Orientation	Houston
December 28	Release of NSBRI Research Announcement 99-02: Formation of New Research Teams	N/A
January 10-13, 2000	NSBRI RETREAT	Del Lago, TX
January 17-21	Russian Countermeasure Course	Houston
February 22	Release of NSBRI Research Announcement 00-01: Expansion of Current Research Teams	N/A
March 1-2	External Advisory Council Meeting	Boston
March 23	Board of Directors Meeting	Houston
April 4-5	Artificial Gravity Workshop	Houston
May 5	Proposal Due Date for Research Announcement 99-02	N/A
May 10-11	Meeting with Officials of the Canadian Space Agency	Toronto
June 13-15	WORKSHOP on Multicultural and International Issues in Space Flight Research & Health Care	Houston
June 19	Proposal Due Date for Research Announcement 00-01	N/A
June 19	Release of Special Program Announcement 00-02: Expansion of Education & Public Outreach Activities	N/A
June 20	NSBRI Board Strategic Planning Subcommittee Meeting	Dallas
June 22 – August 8	Peer Panel Meetings: 12 Study Sections	Washington
August 24-25	NSBRI Program Planning Meeting	Laurel, MD
September 12-13	External Advisory Council Meeting	Laurel, MD
September 15	Proposal Due Date for Research Announcement 00-02	N/A
September 20	NSBRI Board Strategic Planning Subcommittee Meeting	Houston
September 20	NSBRI Board Audit Subcommittee Meeting	Houston
September 21	Board of Directors Meeting	Houston

the eight original research areas. This announcement is included as Appendix H. It resulted from eight workshops from October through December 1999 and focused on the risks and critical questions identified in NASA's critical path roadmap. Workshop attendees are included as Appendix I of this report.

As reported in last year's annual report, the four new research areas are:

- Integrated Human Function – Developing a sufficient understanding of human function (from molecules to systems) to enable a reliable evaluation and prediction of an astronaut's safety and functional capacity;
- Neurobehavioral and Psychosocial Factors – Focusing on research that will ensure that astronaut neurobehavioral health is maintained during prolonged missions, that astronaut performance capability is facilitated by appropriate habitat and human-systems interfaces, and that crew functioning is effectively optimized;
- Nutrition, Physical Fitness and Rehabilitation – Integrating nutritional, physical fitness, and pharmacological approaches into a unified countermeasure protocol that maintains mental and physical health during flight and speeds up recovery after flight
- Smart Medical Systems – Developing new and advanced concepts of medical monitoring, diagnostic and therapeutic systems, with the ultimate goal of developing a smart, integrated medical system that would assist in the delivery of quality health care on an exploration-class mission.

Two hundred and eight-one different proposals were received in response to these two announcements. (N.B. One proposal was submitted twice, once to each announcement, leading to an early report of 282 proposals submitted.) Proposals were reviewed by 12 peer panels assembled by NSBRI's peer review contractor. Each panel focussed on one of the 12 research areas. Lists of the panelists are provided in Appendix J. Also included in Appendix I are the review guidelines provided by the contractor to the panel members. As explained in those guidelines, each proposal received an evaluation and was placed in one of five qualitative categories: excellent, good, weak, not scored, and not recommended for further consideration. Proposals in the first three categories received a numerical score ranging from 1 to 100: excellent (85-100), good (65-84), and weak (1-64). Note that weak proposals would not be considered for funding, but that excellent and good proposals would be included in the competitive range and be considered further. Appendix K contains a table showing the review scores in each research area and a bar graph showing the distribution of scores of all 281 proposals evaluated by the panels. Selection results will be reported in next year's annual report, as the NSBRI received no FY 2001 budget during FY 2000.

4.0 KEY PERSONNEL

During FY 2000, the senior Institute management team (Bobby R. Alford, M.D., Chairman of the Board and Chief Executive Officer, Laurence R. Young, Sc.D., Director, and Ronald J. White, Ph.D., Associate Director) did not change. However, the Board of Directors, in their March 2000 meeting, determined that the Institute's activities and programs were on the verge of growing considerably and that it was in the best interests of the Institute to have a full-time director resident at NSBRI's Headquarters in Houston. Since, Dr. Young wished to remain at MIT, the Board decided to conduct a formal search for a new director and hired the search firm

of Heidrick & Struggles, Inc. to assist them in their search. NASA has agreed that this step is in the best interests of the NSBRI.

During FY 2000, all eight of the team leaders have continued to function as the research "program directors." However, in the recompetition for FY 2001 funding, the team leader of the Muscle Alterations and Atrophy Team (R. Schwartz) failed to achieve a score in the competitive range. He will be replaced by Kenneth Baldwin, University of California, Irvine, in FY 2001. In addition, one of the tentative new team leaders identified in the research announcement (W. Evans) also failed to obtain a score in the competitive range and he will be replaced by Joanne Lupton of Texas A&M in FY 2001.

No principal investigator changes were made in FY 2000. In the area of Human performance, Sleep and Circadian Rhythms, Dirk-Jan Dijk, a principal investigator, moved from Harvard Medical School to the University of Surrey. A number of co-investigator institutional changes were made, but these changes did not negatively impact the research in progress.

5.0 MANAGEMENT PLAN

The original management plan described in the proposal to establish the NSBRI has continued to serve the Institute's needs during the Institute's third full year of operation and has not been modified. For convenience, the management structure is shown in Figure 1, adopted from the original proposal.

Current membership of the NSBRI ***Board of Directors*** is shown in Tables 2 and 3. This Board met in Houston twice during FY 2000 (see Table 1). Three members of last years Board were changed during this year. Richard Ewing of Texas A&M replaced Robert Kennedy who left Texas A&M. Martin Fettman replaced Bernard Cohen who relinquished his chairmanship of the External Advisory Council and *ex officio* seat on the Board because of conflict of interest, since his institution became a consortium member. Martha Gray of MIT replaced Arnold Weinberg who retired from the Board for health reasons. Because of the addition of five new consortium institutions, ten new members were added to the Board and these are shown in Table 3.

Current membership of the NSBRI ***External Advisory Council*** is shown in Table 4. This year, significant membership rotation occurred. Bernard Cohen, Antonio Gotto, Michael Holick, Ann Kennedy, Martin Kushmerick, Donald Marsh and Richard Satava all left the Council after two and a half years of service. Joining the Council were Leon Alkalai, J. A. Anderson, Ruth Benca, Hal Broxmeyer, Thomas Budinger, Dennis Charney, Victor Convertino, Thomas Fleisher, Amy Kronenberg, Charles Nemeroff, Lawrence Palinkas and F. Eugene Yates. The Council met twice in FY 2000, once in Boston in March and once in Laurel, Maryland in September.

NSBRI's ***Board of Scientific Counselors (BSC)*** did not change in FY 2000 and had an oversight role in the summer peer reviews that were carried out. A number of BSC members removed themselves from the reviews because they had submitted proposals themselves as investigators, but others seved as panel chairs of one of the 12 review panels. Rotation will occur in BSC membership in FY 2001.

Figure 1. Originally Proposed NSBRI Structure.

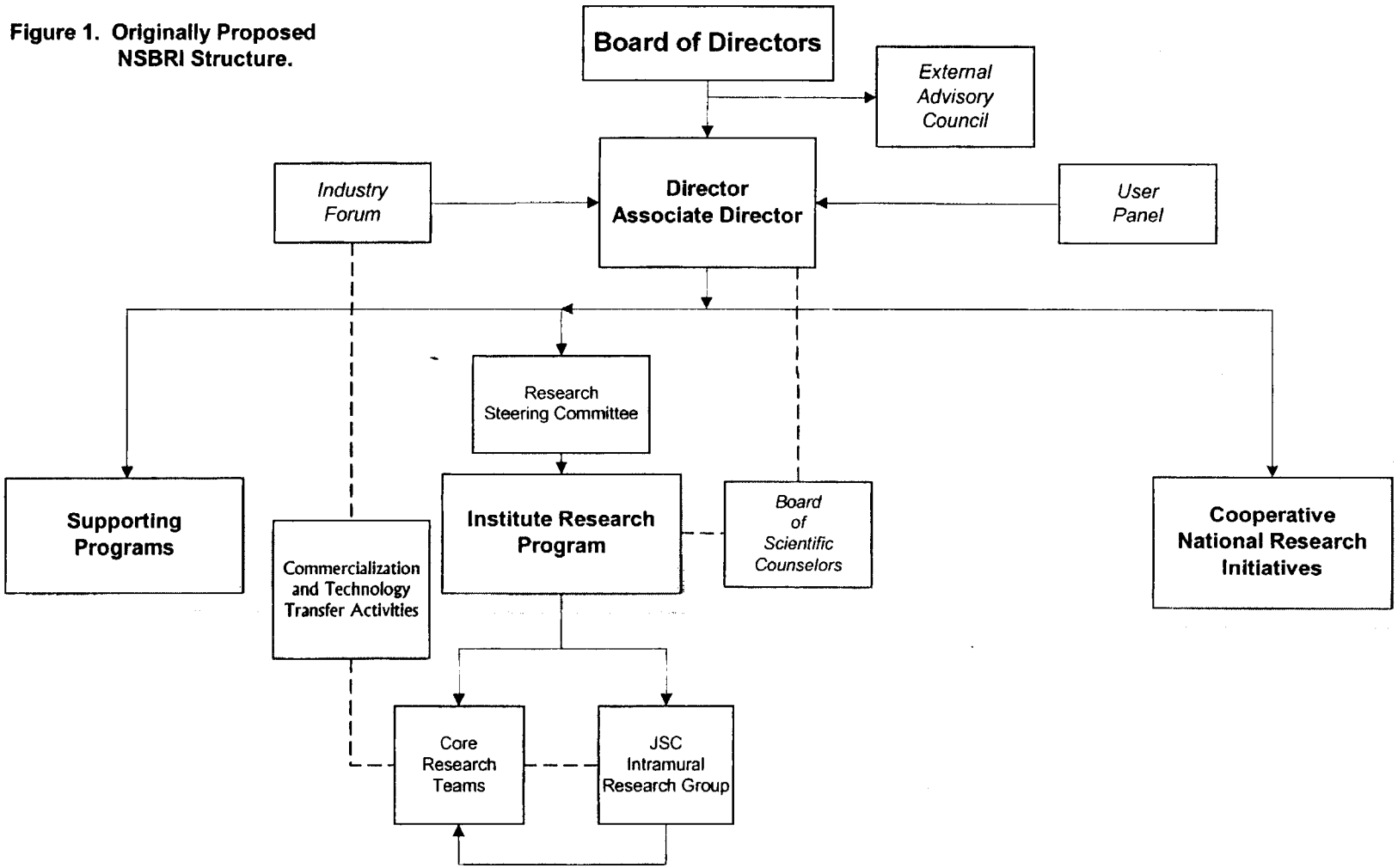


Table 2. National Space Biomedical Research Institute ***BOARD OF DIRECTORS*, Original Consortium Members.**

Bobby R. Alford, M.D. (Chairman) Executive Vice President and Dean of Medicine Baylor College of Medicine	**William L. Allen** Editor National Geographic Magazine	**Joseph V. Bonventre, M.D., Ph.D.** Co-Director, Harvard-MIT Division of Health Sciences & Technology Harvard Medical School
James F. Buchli Space Station Program Manager United Space Alliance	**Aaron Cohen** Zachry Professor of Mechanical Engineering Texas A&M University	**Michael DeBakey, M.D.** Chancellor Emeritus Baylor College of Medicine
Richard E. Ewing, Ph.D. Vice President for Research Texas A&M University	**Martin J. Fettman, D.V.M., Ph.D.** *(ex officio)* Associate Dean for the Professional Veterinary Medical Program Colorado State University	**Martha L. Gray, Ph.D.** Co-director MIT/Harvard Division of Health Sciences and Technology MIT
E. Nigel Harris, M.D. Dean and Senior Vice President for Academic Affairs Morehouse School of Medicine	**Richard J. Johns, M.D.** Distinguished Service Professor of Biomedical Engineering Johns Hopkins University School of Medicine	**Dennis Kasper, M.D.** Executive Dean of Academic Programs Harvard Medical School
Joseph P. Kerwin, M.D. Senior Vice President Wyle Laboratories	**Steven Knapp, Ph.D.** Provost and Vice President for Academic Affairs Johns Hopkins University	**Jordan Konisky, Ph.D.** Vice Provost for Research & Graduate Studies Rice University
J. David Litster, Ph.D. Vice President for Research & Dean of Graduate Education MIT	**Larry McIntire, Ph.D.** E.D. Butcher Professor of Chemical Engineering Rice University	**Francis D. Moore, M.D.** Moseley Professor of Surgery, Emeritus Harvard Medical School
James W. Patrick, Ph.D. Vice President and Dean of Research Baylor College of Medicine	**Walter W. Sullivan, Ph.D.** Vice President of Operations and Planning Morehouse School of Medicine	**W. Dalton Tomlin** (Secretary/Treasurer) Senior Vice President & General Counsel Baylor College of Medicine
Torsten N. Wiesel, M.D. President Emeritus Rockefeller University		**Laurence R. Young, Sc.D.** *(ex officio)* Institute Director

Membership in the ***User Panel*** was stable in FY 2000, but the panel did not meet as a group, because countermeasure ideas had not yet developed to the point where User Panel review was appropriate. Discussion did take place from time to time between individual members of the User Panel and individual investigators, particularly at the Institute retreat.

In January 2000, the second biennial NSBRI retreat was held at the Del Lago Conference Center just north of Houston. The agenda for this retreat is included as Appendix L. As stated in the original NSBRI proposal, "The purposes of these retreats are to enable and encourage dialogue and synergy across the teams, to foster critical examination of research pathways, and to enable technology transfer to and from the private sector. This process should lead to a dynamic, synergistic research program appropriate to the NSBRI." The retreat was very successful, with attendance of over 300 investigators, advisors, students, and NASA personnel.

Table 3. National Space Biomedical Research Institute ***BOARD OF DIRECTORS*, New Consortium Members.**

Carl W. Anderson, Ph.D. Chairman Department of Biology Brookhaven National Laboratory	**Thomas E. Andreoli, M.D.** Professor and Chairman Department of Internal Medicine University of Arkansas College of Medicine	**Robert Berne, Ph.D.** Vice President for Academic Development New York University
Alfred P. Fishman, M.D. Senior Associate Dean Office of Program Development University of Pennsylvania Health System	**Glen N. Gaulton, Ph.D.** Vice Dean for Research and Research Training University of Pennsylvania School of Medicine	**Alvin L. Kwiram, Ph.D.** Vice Provost for Research University of Washington
Peter Paul, Ph.D. Deputy Director, Science and Technology Brookhaven National Laboratory	**Mary R. Rifkin, Ph.D.** Dean for Academic Affairs Mount Sinai School of Medicine	**Robert L. Van Citters, M.D.** Professor and Dean Emeritus University of Washington School of Medicine
I. Dodd Wilson, M.D. Executive Vice Chancellor Dean, College of Medicine University of Arkansas for Medical Sciences		

Table 4. National Space Biomedical Research Institute ***EXTERNAL ADVISORY COUNCIL.***

Martin J. Fettman, D.V.M., Ph.D. (Chairman) Associate Dean for the Professional Veterinary Medical Program Colorado State University	**Leon Alkalai, Ph.D.** Director, Center for Integrated Space Microsystems Jet Propolsion Laboratory	**J. A. Anderson, Ph.D.** Professor of Cognitive & Linguistic Sciences Brown Univesity
Ruth Benca, M.D., Ph.D. Professor and Associate Chair Department of Psychiatry University of Wisconsin	**Hal E. Broxmeyer, Ph.D.** Chairman and Professor Walther Oncology Center Indiana University School of Medicine	**Thomas F. Budinger, M.D., Ph.D.** Professor and Chair, Department of Bioengineering Lawrence Berkeley National Laboratory
Dennis S. Charney, M.D. Chief of Mood and Anxiety Disorders Research Program National Institute of Mental Health	**Victor A. Convertino, Ph.D.** Research Physiologist U.S. Army Institute of Surgical Research	**Thomas A. Fleisher, M.D.** Chief, Department of Laboratory Medicine National Institutes of Health
Michael N. Gould, Ph.D. Professor of Human Oncology University of Wisconsin	**Amy Kronenberg, Sc.D.** Group Leader Radiation Biology and Environmental Toxicology Lawrence Berkeley National Laboratory	**Robert Y. Moore, M.D., Ph.D.** Professor and Chairman of Neurology University of Pittsburgh
Charles B. Nemeroff, M.D., Ph.D. Professor and Chairman of Psychiatry Emory University	**Lawrence A. Palinkas, Ph.D.** Professor, Family and Preventative Medicine University of California, San Diego	**Danny A. Riley, Ph.D.** Professor of Cell Biology and Anatomy Medical College of Wisconsin
Irwin H. Rosenberg, M.D. Professor of Medicine and Nutrition Tufts University	**M. Rhea Seddon** Assistant Chief Medical Officer Vanderbilt University Medical Center	**Warren K. Sinclair, Ph.D.** President Emeritus National Council on Radiation Protection & Measurement
Ronald J. White, Ph.D. (*ex officio*) Institute Associate Director	**Victor J. Wilson, Ph.D.** Professor of Neurophysiology Rockefeller University	**Thomas J. Wronski, Ph.D.** Professor of Physiological Sciences University of Florida
Bill J. Yates, Ph.D. Assistant Professor of Otolaryngology and Neuroscience University of Pittsburgh	**F. Eugene Yates, M.D.** Professor of Medicine University of California, Los Angeles	**Laurence R. Young, Sc.D.** (*ex officio*) Institute Director

Table 5. Membership in the National Space Biomedical Research Institute ***INDUSTRY FORUM.***

Boeing Space and Communications Group
The Charles Stark Draper Laboratory
Information Dynamics, Inc.
Lockheed Martin Engineering & Science Services
Michigan Biotechnology International
Payload Systems, Inc.
Roche Laboratories, Inc.
Silicon Graphics, Inc.
Southwestern Bell
United Space Alliance
Veridian
Wyle Laboratories

6.0 COMMERCIALIZATION, EDUCATION AND PUBLIC OUTREACH

During FY 2000, the ***Industry Forum*** (Table 5) membership changed slightly. Merck Research Laboratories resigned its membership, but Roche Laboratories, Inc. was added to the group. Raytheon Company also resigned its membership. Activities of the Forum included meeting with all of the Team Leaders in March at the External Advisory Council meeting and presenting a briefing entitled "Moving Countermeasures Forward." This briefing was designed to assist the team to efficiently and effectively develop ideas into applications for use in space and to assist them in applying their work to Earth-bound problems. In addition the Forum prepared a white paper on "Management and Protection of Intellectual Property" for use by the teams as they develop commercial products from their research projects.

The Education and Public Outreach Team supports the NSBRI's mission by ensuring open involvement in the Institute's activities by the scientific community, industry and the public at large, and by ensuring a robust exchange with NASA. Activities target multiple and diverse populations and aim to:

- inform a large community about NSBRI activities;
- attract young people to careers in science, engineering and medicine;
- promote excellence and innovation in America's science education system;
- increase scientific literacy among teachers, students, their families, and the public at large; and
- create public awareness and appreciation of the opportunities and benefits of NSBRI's space biomedical research.

Through a variety of innovative programs, space research activities and discoveries are transferred to teachers at levels K-undergraduate, students and the general public.

The National Research Council's Committee on Undergraduate Education has challenged the scientific community and institutions of higher learning to provide opportunities for professional collaborations that create innovative inquiry-based, multidisciplinary courses and instructional materials; to use the most sophisticated multimedia capabilities to disseminate new materials; and to develop a seamless pipeline of minority-group science students. The NSBRI has embedded this challenge in its education and public outreach mission.

The NSBRI's educational programs respond to the needs of the full educational spectrum. The three primary areas of focus are:

- creation of supplementary educational materials for elementary and secondary school teachers and students;
- development of educational opportunities and courses for undergraduate students and teachers; and
- production of educational resources for school audiences and the general public using a variety of electronic media, such as computer-based multimedia (including the World Wide Web), radio and television.

To guide the development of NSBRI educational materials, the team identified relevant content areas described within the National Science Education Standards that can be taught by using space life sciences as unifying themes. Current outreach activities are being led by teams at three consortium institutions: Morehouse School of Medicine, Texas A&M University and Baylor College of Medicine.

A Special Program Announcement for NSBRI Consortium Institutions (NSBRI 00-02) was issued June 19, 2000, to solicit proposals to expand the education and public outreach activities. This announcement is included as Appendix M. The Institute received 18 proposals in respoonse to this announcement. Following peer review, a selection of new projects will be made early in FY 2001.

Project activities conducted in FY2000 by the three institutions follow.

Morehouse School of Medicine. Morehouse's Education and Public Outreach projects aim to establish and maintain partnerships to create multimedia educational materials that bring space biomedical sciences to America's schools and that communicate the benefits of space exploration and NSBRI technology spin-offs to the lay public.

Teacher Fellowship Program. This program is in collaboration with the DeKalb school system's Fernbank SpaceStation and Georgia State University SECME programs. Fellows complete a training program and become a member of a multidisciplinary case writing team that prepares a problem-based case. The 1999-2000 Teacher Fellow was Terri Brown, a biologist who directs the Fernbank electron microscopy laboratory. The DeKalb school system provided release time for her fellowship at Morehouse. A student intern was also selected to work with the materials development team. This intern position is designed to ensure that the case dialogue is in sync with the culture of today's media-literate students and will hold students' attention.

Brain in Space Teacher's Guide. A color version of the NASA-approved Neurolab curriculum, *The Brain in Space,* is now available on the NSBRI Web site. The curriculum was adapted as a

training module by the Massachusetts Partnership for Learning Mathematics and Science program and the Atlanta Public Schools Systemic Initiative. A partnership grant has been earmarked for the production of two space-neuroscience problem-based cases with Atlanta Public School teachers.

College Course. *The Human Body in Space,* a pilot program being taught at Spelman College, gives college students an appreciation of the historic science challenges of space flight, knowledge of the space environment and an understanding of biological adaptations related to weightlessness. Drawing from several disciplines, the course develops a framework for understanding the biomedical challenges to be understood if humans are to effectively explore and develop space and some of the cultural implications of long-term human space flight and habitation of the International Space Station. The course engages students in lectures, discussions, individual research papers and oral presentations.

This pilot course is the basis for designing an NSBRI national curriculum on the human body in space. The curriculum will include modules and video pieces appropriate for college-level students. Enrollees also create a pipeline of students interested in graduate education in biomedical sciences and the NSBRI Summer Research Program at Morehouse.

Summer Research Program. The program encourages women and minority-group students to pursue careers in medicine and biomedical research. In FY00, the program enrolled four students selected from a competitive, national applicant pool. Participants engage in a research-intensive internship at Morehouse, learn about space biomedical research from an eminent space sciences guest lecturer and meet role models involved in space biomedical research. Each student is required to undertake a well-defined research project approved by a faculty mentor. At the end of the program, participants must write a research report and present their findings to faculty and fellow students at a school-wide event. Their presentations are filmed as archival footage for NSBRI.

To date, 13 students have participated in the intensive 12-week research program that is part of Morehouse's Neuroscience Institute Summer Program and the Morehouse/NASA Space Medicine Life Sciences Research Center. Follow up data on the students show that they are currently engaged as follows:

Medical Training:	
Loma Linda Medical School	1 student
Morehouse School of Medicine	4 students
Pre-Medicine/Science Majors:	
Florida A&M University	2 students
Xavier College	1 student
University of Houston	2 students
Morris Brown College	1 student
Secondary Science Teacher	1 student
Undecided	1 student
Total	**13 students**

NSBRI Introductory Video. Morehouse and the Atlanta Educational Tele-communications Collaborative, Inc., produced a 10-minute NSBRI introductory video intended for use with multiple audiences, including student groups, teachers, the public and industry. The video communicates NSBRI's mission, provides a brief overview of the science and puts a "human face" on the science so that lay audiences can understand the impact NSBRI research will have on their daily lives. The video highlights the relationship between NSBRI's research objectives

and NASA's goal of long-term space travel and communicates the NSBRI Education and Public Outreach Program's vision.

NSBRI Archives. Morehouse established a state-of-the-art, AVID film-editing suite to support the creation of broadcast-quality video products and to support the development of a comprehensive space life sciences film archive. The archives contain all footage associated with the Morehouse-Neurolab Education Program, including raw footage from the Neurolab documentary; a five-day teacher-training workshop on the brain; complete text and film of the Human Body in Space course; footage of the summer interns presentations; and footage from various NSBRI events. In FY00, the Archive has reproduced footage for several NSBRI consortium members, Atlanta and DeKalb public schools, and the SciTrek and Fernbank museums. At the request of the Headquarters Communications and Outreach Office, footage was also provided to the Discovery Channel, ZDF-German TV and RDF Television-London.

Texas A&M University. Texas A&M's scope of work for this period involved two program thrusts: the teacher academy and the electronic resources – Web site development. Robert James directed the teacher academy program and George Jessup directed the electronic resources – Web site development effort. Jon Denton provided overall program management.

Teacher Academy. The goal of this project is to prepare Master Teachers to assist their peers in infusing cutting-edge, space-based science activities in K-12 classrooms. Nineteen teachers participated in the summer institutes held in 1997 and 1998. In FY 00, the Academy staff monitored and encouraged the work of the 19 Teacher Academy Fellows. Follow-up activities included electronic contact with the teachers and participation at the NASA Johnson Space Center's Inspection 99. Participants reported classroom activities and outreach results. Teachers reported teaching units ranging from two weeks to three months in length. Seventeen of the nineteen teachers reported doing experiments in at least one of the research areas with one-third doing at least two.

Texas A&M also has implemented "Mission to Mars" activities in the university's teacher certification programs. Pre-service teachers, working in cross-disciplinary teams of 5 or less, assembled resource units focusing on the science, technology and society themes involved in manned space flight.

Electronic Resources – Web Site Development. In FY 00, project staff from Texas A&M worked with the NSBRI Headquarters Communications and Outreach Office to re-design the NSBRI Web site. The project involved development of a new look for the site, making it easier to navigate and more visually pleasing to the user. In addition, a secure site within the NSBRI public Web site was developed to allow key leadership groups, advisory council members and researchers to access reports and information easily. Currently, there are over 70 documents, reports and presentations from the different research teams at this site. Texas A&M continues to serve as Webmaster for the site while Headquarters Communications and Outreach is responsible for the content. All Education and Public Outreach curriculum materials can now be obtained through the site.

Through applications of multimedia technologies, Texas A&M is preparing electronic curricular materials for K-12 science and health classrooms to promote students' understanding of medical and policy issues associated with sustained space flight to Mars. Texas A&M's NSBRI educational Web site activities emphasize:

- Establishing electronic on-line secondary school human physiology course;

- Refining the Education and Public Outreach page, so that educational and supporting resources provided by all NSBRI partners are available in a centralized location;
- Informing and promoting these electronic space science curricular resources to educational professional associations systemic science initiatives in various states, state education offices and federal regional technology in education consortia;
- Producing electronic multimedia NSBRI researcher profiles to personalize space science research efforts;
- Developing Web-based instructional modules associated with each NSBRI research area, and;
- Developing NSBRI instructional elements for teacher education students in science education

NSBRI Poster for NSTA. Texas A&M produced a poster highlighting the NSBRI's Web site that was distributed to participants at the National Science Teachers Association meeting. More than 9,000 copies were distributed at the meeting along with other NSBRI Education and Public Outreach materials.

Baylor College of Medicine. Baylor activities involve the production, evaluation and distribution of upper elementary activities guides that foster the acquisition of inquiry skills and science content knowledge. Another program, Baylor's Radio Healthline, generates public awareness and enthusiasm for space biomedical research and promotes understanding of the relevance of NSBRI research to the treatment of patients suffering from diverse ailments on Earth.

From Outer Space to Inner Space Activities Guides. Baylor has continued the development of a series (From Outer Space to Inner Space) of challenging interdisciplinary units that help upper elementary and middle school students and teachers develop inquiry skills and content knowledge in physical, earth/space and life science related to NSBRI research areas. The project partners scientists and educators in the design, production, evaluation and dissemination of age-appropriate, supplemental curriculum modules based on space biomedical themes for students and teachers in grades K-8. In FY 00, the first two units were completed.

Sleep and Daily Rhythms, the first unit in the series, examines sleep and circadian rhythms and was completed in Fall 1999. The Activities Guide is available free-of-charge and has been received enthusiastically from teachers throughout the U.S. More than 10,000 copies have been disseminated at state and national workshops (including the National Science Teachers Association) and as part of an NSBRI introductory education packet sent to more than 6,000 elementary and middle schools in Arkansas, Pennsylvania, New York and Washington (the new consortium-member states). The unit can be downloaded from the NSBRI Web site. With the assistance of the Headquarters Communications and Outreach Office, the unit was highlighted in feature articles in the Los Angeles Times, the Houston Chronicle and the Texas Medical Center News.

The second unit, *Muscles and Bones*, was field-tested with 340 students in Houston-area schools during summer 2000. This 40-page unit teaches students about gravity and the musculo-skeletal system; growth and development of bones and muscles; and the physics of the skeletal system. Examination of student work and pre-/post-assessments demonstrated increased student learning about bone/muscle structure, how exercise strengthens bone and muscle and which foods contribute to strong bone/muscle. The unit includes ten activities.

Radio Healthline Series. Healthline stories on NSBRI-related topics are being produced and disseminated bimonthly through Baylor's Office of Public Affairs. The news-format stories are distributed free-of-charge to more than 2,900 stations and are made available in both English and Spanish. The stories also are available on the NSBRI's Web site. Stories produced in FY 00 are listed in Table 6.

Table 6. FY 00 Radio Healthline Stories

Date	Title	NSBRI Faculty Interviewed
October 1999	Working with Russians	John Lednicky, Ph.D.
December 1999	Space Machines	Vincent Pisacane, Ph.D.
February 2000	Radiation	John Dicello, Ph.D.
April 2000	Motion Sickness	Charles Oman, Ph.D.
June 2000	Perfect Harmony	JoAnna Wood, Ph.D.
August 2000	Math Model	Larry Young, Sc.D.

Support Activities for Public Outreach. Baylor's outreach team has provided support for a number of activities designed to generate public awareness and support of NSBRI activities. Each of these activities is outlined briefly below.

- *Graphic design of a new outreach booklet about the NSBRI highlighting the individual research team activities, the expanded research areas and the NSBRI's supporting activities.* Team icons were developed for the four proposed research areas, and graphics design talent was provided for this booklet developed by the Headquarters Communications and Outreach Office.
- *Continued dissemination of activity-based educational poster for K-6 students, teachers and parents.* Requests for the poster, created in Spring 1999, continue to be filled. The activity poster contains basic information about NSBRI research, as well as activities for students.

Presentations given by NSBRI Education and Public Outreach Team Members.

- Clipper, M., W. Sharpe, and M. MacLeish. *NSBRI Education and Public Outreach: A Model for Developing Alzheimer's Disease Education and Public Outreach.* Center on Aging, Emory University, Atlanta, GA.

- Clipper, M., W. Sharpe, and M. MacLeish. *NSBRI Education and Public Outreach Partnerships: A Presentation to Mount Sinai School of Medicine*, Atlanta, GA.

- Clipper, M., W. Sharpe, and M. MacLeish. *NSBRI Education and Public Outeach: Space Life Sciences Education for the Nation.* Kennedy Space Center Scholar Visit, Atlanta, GA.

- Clipper, M. and M. MacLeish. 2000, *Improving Science Literacy and Education Through Space Life Sciences.* 13th Man in Space Symposium, Santorini, Greece, May 20-25.

- MacLeish, M. *NSBRI K-12 Space Life Sciences Content for the DTV ERA.* Atlanta Public Schools DTV Transition Meeting, Atlanta, GA.

- MacLeish, M. *NSBRI-CBN Partnership for Success.* Center for Behavioral Neuroscience, Emory University, Atlanta, GA.

- MacLeish, M. *Telemedicine for Health Delivery.* Halstead Communications, Atlanta, GA.

- MacLeish, M. *NSBRI Science for Problem -based Learning Cases.* Fernbank Museum - Teacher Fellowship Two-week Mini-course, Atlanta, GA.

- MacLeish, M. *NSBRI Science and Space Life Sciences Careers.* MSM-NSBRI Summer Research Program, Atlanta. GA.

- MacLeish, M. *NSBRI Science: In Support of Science Literacy and Education for the Nation.* Senator Mark Hatfield's Visit to MSM, Atlanta, GA.

- MacLeish, M. *NSBRI Science: Science Literacy and Education Spin-offs For The Nation.* Atlanta Press and Broadcasters Meeting, Atlanta, GA.

- Moreno, N., B. Tharp, and R. James. 2000, *To Mars and Back: From Outer Space to Innerspace.* National Science Teachers Association, Orlando, FL, April 5-9.

- Patrickson, J., J. Whittaker, and M. MacLeish. *The Brain in Space.* SECME - Two-day NSBRI Sponsored Teacher Professional Development Sessions, Atlanta, GA.

7.0 INSTITUTE DIVERSITY AND SCIENTIFIC COMMUNITY OUTREACH

Sine the NSBRI's research program was stable in FY 2000, there was virtually no investigator turnover and our investigator list is very much the same as it was last year (see Appendix A). However, in order to encourage investigators at minority institutions to become more competitive in the future, seed money was provided to Morehouse Medical School to enable a few of their investigators to develop pilot data for future projects that would be proposed in response to Institute research announcements. In addition, every attempt was made to provide our research announcements to a wide and diverse group of potential investigators. In fact, NASA was kind enough to provide the mailing list that it uses for announcements related to the life sciences and this list was the backbone of the postal card mailout that informed the community of the availability of the research announcements.

Institute investigators, team leaders, and management continued to reach out to the scientific community through presentations made at symposia and meetings. A partial list of these presentations is provided in Appendix F. Once again, Institute workshops mentioned in other places in this report brought new researchers into contact with the NSBRI and its mission. Preliminary discussions in Toronto with the Canadian Space Agency in May 2000 set the stage for further interaction directed towards developing a cooperative agreement allowing the Canadian scientific community greater ability to participate in NSBRI activities.

This year, as part of its outreach program, the Institute sponsored three students from the Houston Premedical Academy to work for six weeks during the summer at the Massachusetts Institute of Technology. These three minority undergraduate students worked on research projects in the laboratories of Elazer Edelman, Mehmit Toner, and Jeffrey Morgan at MIT and benefited greatly from the positive experience.

8.0 SPECIAL PROJECTS

The *Cooperative Agreement Management Plan* between NASA and the NSBRI enables the partners to undertake special projects outside of the core funding envelope of the NSBRI. During FY 2000, seven new projects were initiated, two previous projects were completed and four were continued. Field testing of the four lesson plan modules described last year under Project 99-3, *Educational Outreach – Field-Testing Of Draft Lesson Plan Modules*, was completed this year. By mutual agreement, it was decided that further Institute planning described in Project 99-5, *Tactical Planning and Integration*, would be deferred pending resolution of reorganization issues at NASA. As described earlier in this report, Project 99-6, *Collaboration with Russia's Institute for Biomedical Problems (IBMP)*, was also completed, with the required reports being submitted according to the agreed upon schedule. In one continuing project, Project 99-7, *Food Scientist, Food Laboratory*, Dr. Vodovotz left the Institute for Ohio State University and was replaced by Dr. Michele Perchonok.

Project 97-3, *National Space Biomedical Research Institute Visiting Scientist/Research Associate Program*, continued to enable young and established university-based researchers an opportunity to work side-by-side with government employees in JSC laboratories. Table 7 provides a list of the participants in this program; all faculty positions are held at Baylor College of Medicine.

Activity continued on Project 98-1, the *NSBRI Data Archive System*, during FY 2000. This project, led by Lora Suther of The Johns Hopkins University Applied Physics Laboratory (APL), was established in FY 98 with the goal of maintaining an appropriate, accessible archive of the data collected through the NSBRI research projects. During this year, a data management plan was developed and adopted by the Institute research teams. This plan describes the NSBRI data archive system, structure, and policies. It is intended to inform the community of what types of products are available and how access is provided. The plan addresses the standards and policies for data collected by the Institute. It delineates the data rights to data collected in research funded by the Institute and specifies the timely delivery of the collected research data and results to the archive. It addresses the data formats, communications methods, and backup requirements, to define a robust data model to meet the Institute's storage and retrieval needs. The data management plan is available on the Institute web site (www.nsbri.org). In addition, a web interface for the Institute data archive has been designed and tested. This interface is compatible with and similar to NASA's Life Sciences Data Archive at Johnson Space Center. It provides the following: face provides the following:

- A master catalog providing links to each of the categories upon which the archive may be searched;
- Search pages and display pages to provide access to experiment information, cataloged digital images, data sets, hardware, information on documents, personnel, information on specimens/subjects;
- Information on each of the NSBRI research teams;
- An overview of the IDAS;
- Links to web pages related to the NSBRI and the IDAS archive;

- Access to the future outreach site to be developed by Morehouse School of Medicine;
- News related to the IDAS;
- Interface via which feedback may be provided to the IDAS team;
- Glossary of related terms;
- Acronym list;
- Full text search of team/project/experiment descriptions; and
- On-line help.

Table 7. Participants in the Visiting Scientist/Research Associate Program for FY 00.

Name	Current Position	JSC Sponsor	Period
Charlotte Adams, Ph.D.	Research Associate	Clarence Sams	1/05/00 – 5/14/00
Tatiana Christian	Senior Engineer	Jerry Goodman *(see Project 00-5)*	5/22/00 -
Johnny Conkin, Ph.D.	Assistant Professor	John Stanford	6/1/98 -
Dominick D'Aunno, M.D.	Assistant Professor	Jan Yelle	11/1/97 -
Laura E. Duvall	Senior Engineer	Thomas Rathjen *(see Project 00-7)*	9/25/00 -
Philip Foster, M.D.	Assistant Professor	John Stanford	10/19/98 -
Victor Hurst, Ph.D.	Research Associate	Jan Yelle	8/23/99 – 6/12/00
Giles Maule, Ph.D.	Research Associate	Clarence Sams	1/24/00 -
Jennifer Novak, Ph.D.	Assistant Professor	Dane Russo *(see Project 00-1)*	1/4/00 -
Michele Perchonok, Ph.D.	Assistant Professor	Dane Russo *(see Project 99-7)*	9/5/00 -
Sudhakar Rajulu, Ph.D.	Assistant Professor	Dane Russo *(see Project 00-3)*	4/17/00 -
Chantal Rivera, Ph.D.	Research Associate	Lakshmi Putcha	5/6/99 – 7/2/00
Lawrence Spector	Senior Engineer	Thomas Rathjen *(see Project 00-6)*	9/25/00 -
Yael Vodovotz, Ph.D.	Assistant Professor	Dane Russo *(see Project 99-7)*	8/31/99 – 6/1/00
Wendy Waters, Ph.D.	Assistant Professor	Jan Yelle	11/24/97 -
Scott Wood	Research Associate	William Paloski	9/14/98 – 9/29/00

A workshop on multicultural and international issues in space flight research and health care was held in July 2000 as part of Project 99-4, *Enabling a Broader Segment of the Population to Explore, Live and Work in Space in the 21st Century*. The workshop report is included as Appendix N.

Project 00-1, *Human Factors Engineering & Operational Habitability*, is a project designed to enable the NSBRI and NASA JSC to develop joint activities related to space habitability and applied human factors research. Development of a more thorough understanding of these research areas, which are not part of the Institute's core research activity, will enhance the Institute's ability to develop future research programs that will provide the critical data necessary to promote human engineering and habitability for space flight. To fulfill the requirements of this project, the NSBRI hired Jennifer Novak, Ph.D. This is a long-term project.

Project 00-2, *Space Human Factors and Habitability Plan*, is a project designed to enable the Institute and NASA JSC to plan, develop, and implement a vigorous, strongly linked operational/research program in habitability and environmental factors. The Habitability and Environmental Factors component of the Bioastronautics Program is responsible for ensuring the safe habitability of all aspects of the physical and biotic environment of human spacecraft. It includes the areas of habitability and human factors described above, as well as those activities related to air and water quality, toxicology and microbiology, radiation and orbital debris. This project should enable the NSBRI and NASA to jointly begin to define critical questions, define the necessary applied research and required support for future mission development and operations. The first activity under this project was a two-day NASA-NSBRI Workshop on Space Human Factors and Habitability was held in Houston on April 18-19, 2000. Appendix O provides athe workshop agenda and a list of participants. The workshop focused on the critical questions that the space human factors and habitability component of the Habitability and Environmental Factors Office at Johnson Space Center must address and recommended a plan to address these questions in a timely way.

Project 00-3, *Director, Anthropometry and Biomechanics Program Development*, was designed to enable the NSBRI and NASA JSC to develop their applied capability in the areas of anthropometry and biomechanics by establishing the position of Director, Anthropometry and Biomechanics Program Development at Johnson Space Center and by hiring a qualified person to fill that position. This person will have oversight for all NASA-sponsored data acquisition and utilization activities in this area and will be responsible for providing essential leadership in the integration of data and information into design standards, hardware development, and program support including making recommendations for and implementing measures necessary to establish a state-of-the-art facility for anthropometric and biomechanical data acquisition and utilization. Sudhakar Rajulu, Ph.D. was hired to fill this position. This is a long-term project.

Project 00-4, *Risk Assessment & Management*, is a project designed to examine the issues related to the risks associated with human space flight using new risk models to be developed. This effort will begin with the risks identified in the Critical Path Roadmap, an integrated, cross-disciplinary strategy to assess, understand, mitigate, and manage the risks associated with long-duration exposure to the space environment. The Critical Path Roadmap includes risks, critical questions, and the defined priorities. The specific deliverables associated with this project are:

- Provide a comprehensive plan for the this effort;
- Provide a comparison of the NASA flight based risks with a comparable set of Earth-based risks, in order to establish a quantifiable, normative base for injury, illness, and mortality;

- Review the NASA/NSBRI, Critical Path Roadmap-defined risks; provide an assessment of the presented risks, based upon probability of occurrence, severity of impact to crew and mission, and mitigation status;
- Quantify risk probability and severity of impact;
- Categorize medical, mission and program risks into a standard format to facilitate analyses and communications;
- Provide cost/benefit analyses; and
- Assist NASA in establishing acceptable levels of risk.

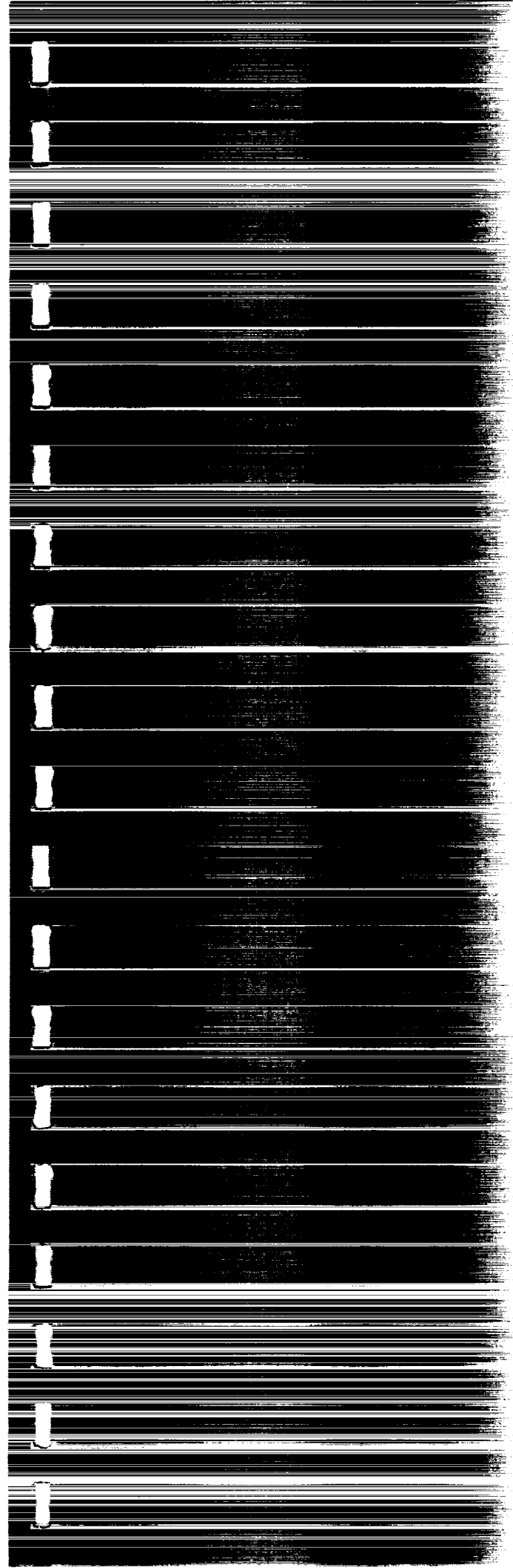
This project will be carried out by the Baylor College of Medicine Risk Management Department working with a consultant team from Marsh, Inc. and Actuarial Research Group.

Project 00-5, *Acoustics Specialists, Operational Habitability Project (OHP)*, is designed to establish NSBRI Visiting Scientist Positions for technically, academically qualified and experienced acoustics experts to work at or consult with NASA JSC. Applied acoustics is very important for the International Space Station and will be important in designing an exploration mission of the future. These experts will guide the major acoustics work necessary to support the Operational Habitability effort including: mission planning and implementation support, data collection and analysis, lessons learned identification, assessments, interface and contact maintenance, and project management. To carry out this project, the NSBRI hired Tatiana Christian and retained Punan Tang, Ph.D. as a consultant.

Project 00-6, *Leadership of Human Factors Research & Analysis Element of the Space Human Factors and Habitability Office*, is a project designed to enable the NSBRI to develop special strength in this area and to attract a qualified and experienced task coordinator and science facilities leader to the Bioastronautics Office at Johnson Space Center. This person will review candidate research activities and make recommendations to NASA concerning their relevance and feasibility and will advise NASA on measures necessary to maintain JSC's facilities in this area as state-of-the-art. To carry out this project, the NSBRI hired Lawrence Spector.

Project 00-7, *Leadership of Stowage and Housekeeping Research & Analysis Element of the Space Human Factors and Habitability Office*, is a project designed to enable the NSBRI to develop special strength in this area and to attract a qualified and experienced crew systems engineer and technical leader to the Bioastronautics Office at Johnson Space Center. This person must develop an expert's knowledge of stowage and housekeeping facets of spacecraft habitability management and provide technical leadership in this area. To carry out this project, the NSBRI hired Laura Duvall.

Finally, in preparation for a potential future space flight project related to artificial gravity, the NSBRI and NASA jointly sponsored a workshop on this subject. Appendix P contains a copy of that report.





NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Principal and Co-Investigator List
FY 2000

Australian Antarctic Division: Lugg, D. J.
Baylor College of Medicine: Alfrey, C. P., Appel, S. H., Butel, J. S., Cohen, H. S., Conner, M. E., Demayo, F. J., Epstein, H. F., Goldberg, J., Hamilton, S. L., Kanwar, S., Keitel, W. A., LeBlanc, A., Lednicky, J. A., Ling, P. D., Mosier, D. R., O'Malley, B. W., Reid, M. B., Rice, L., Rosenblatt, H. M., Schneider, M. D., Schwartz, R. J., Shearer, W. T., Smith, C. L., Smith, C. W., Smith, E. O., Smolen, J. E., Trial, J., Weigel, N. L., Wood, S.
Boston University: Oddsson, L.
Brooklyn College: **Raphan, T.**
Dartmouth College: **Taube, J. S.**
Harvard Medical School: Abdellatif, M., Brown, E., Brown, E. M., Cajochen, C., Czeisler, C. A., Goldberg, A. L., Jewett, M. E., Krebs, D. E., Kronauer, R. E., Lorell, B.H., Luithardt, H. H., Mullen, T. J., Mullington, J. M., Ramsdell, C. D., Ronda, J. M., Rosenthal, N. A., Sheynberg, N., Wall, C. C., Williams, G. H., Wright, K. P.
Johns Hopkins University: Beck, T. J., Berkowitz, D., Bryden, W. A., Charles, H. K. Jr., Cotter, R. J., Dicello, J. F., Fainchtein, R., Fedarko, N. S., Feldmesser, H. S., Gold, R. E., Goldsten, J. O., Griffin, C. A., Huso, D.L., Kinnison, J. D., Mann, J., Maurer, R. H., Minor, L. B., Novak, G. R., O'Malley, B. W. Jr., Piantadosi, S., Pisacane, V. L., Potember, R. S., Ricart, R., Risby, T. H., Roth, D. R., Ruff, C. B., Shelhamer, M. J., Shoukas, A. A., Williams, J. R., Zee, D. S., Zhang, Y.
Loma Linda University Medical Center: Gridley, D. S., Moyers, M. F.
M. D. Anderson Cancer Center: Lee, B. N., Reuben, J. M.
Massachusetts Institute of Technology: Cohen, R. J., Kamm, R. D., Mark, R. G., Newman, D. J., Oman, C. M., Schaffner, G., Sherman, D. A., Szolovitz, P., Young, L. R.
Mayo Clinic: Turner, R. T.
Morehouse School of Medicine: Thierry-Palmer, M.
NASA Johnson Space Center: Bloomberg, J. J., Cucinotta, F. A., Paloski, W. H., Pierson, D. L., Sams, C., Shackelford, L., Yelle, J. M.
NCRR/NIH: Strandberg, J. D.
Russia: Larina, I.
SmithKline Beecham Pharmaceuticals: Suva, L. J.
Texas A&M University: Armstrong, R. B., Bloomfield, S. A., Braby, L. A., Delp, M. D., Hogan, H.A., Sinden, R. R.
Uniformed Services University of the Health Sciences: Shapiro, J. R.
University of California, Irvine: Baldwin, K. M.
University of California, Santa Barbara: Beall, A. C.
University of Florida: Byrne, B. J.
University of Houston: Fox, G. E., Willson, R. C.
University of Missouri: Booth, F. W.

University of Pennsylvania: Dinges, D. F., Maislin, G., Rogers, N., Szuba, M. P., Van Dongen, H. P.
University of Surrey Guildford: Dijk, D., Lockley, S. W.
University of Texas, Houston: Oden, Z. M.
University of Washington: Ochs, H. D.
University of Wisconsin: Howard, S. P.
Washington Hospital: Schultheis, L. W.
Wright State University: Shebilske, W. L.
York University: Howard, I. P.



NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

CORE RESEARCH PROGRAM YEARS 1, 2 AND 3

October 1, 1997 – September 30, 2000
Updated October 1, 2000

NSBRI RESEARCH PROGRAM
BONE LOSS

Team Leader: Shapiro, J. R. — **Uniformed Services University of the Health Sciences (USUHS)/ Walter Reed Army Medical Center**

O'Malley, B. W.	**PI**	**Baylor**	**Novel Receptor-Based Countermeasures to Microgravity-Induced Bone Loss**	**10**
Brown, E. M.	CO-I	Harvard		
Bloomfield, S. A.	CO-I	Texas A&M		
Smith, C. L.	CO-I	Baylor		
Weigel, N. L.	CO-I	Baylor		
Bloomfield, S. A.	**PI**	**Texas A&M**	**Bone Blood Flow During Simulated Microgravity: Physiological and Molecular Mechanisms**	**12**
Delp, M. D.	CO-I	Texas A&M		
Hogan, H.A.	CO-I	Texas A&M		
Suva, L. J.	CO-I	SmithKline Beecham		
Turner, R. T.	CO-I	Mayo Clinic		
Schultheis, L. W.	**PI**	**Wash. Hosp.**	**The Effects of Partial Mechanical Loading and Ibandronate on Skeletal Tissues in the Adult Rat Hindquarter Suspension Model of Microgravity**	**14**
Bloomfield, S. A.	CO-I	Texas A&M		
Fedarko, N. S.	CO-I	Hopkins/SOM		
Ruff, C. B.	CO-I	Hopkins/SOM		
Shapiro, J. R.	CO-I	USUHS		
Thierry-Palmer, M.	CO-I	Morehouse		
Ruff, C. B.	**PI**	**Hopkins/SOM**	**Skeletal Structural Consequences of Reduced Gravity Environments**	**15**
Beck, T. J.	CO-I	Hopkins/SOM		
Newman, D. J.	CO-I	MIT		
Oden, Z. M.	CO-I	UT-Houston		
Schaffner, G.	CO-I	MIT		

BONE LOSS TEAM
PROGRAM EXECUTIVE SUMMARY

The National Space Biomedical Research Institute was created in 1997 with the intent of developing new and effective countermeasures to health risks anticipated during prolonged exposure to microgravity. Bone loss during extended spaceflight is a major risk to astronaut health and safety. The extent of bone loss has been documented based on bone density measurements obtained before and after several flights of varying duration. These include data obtained from the Gemini series, Skylab flights, Souyez flights and more recently from the Mir cosmonauts. In addition to bone density measurements, variations in serum and urine biomarkers of bone turnover and calcitropic hormones have been assayed. These results indicate that bone loss approximates 1-2% per month with sizable variation by individual and bone site measured, that both increased bone resorption and decreased bone formation contribute to the decrease in bone mass, and that it has not been possible to minimize bone loss with various exercise programs or dietary supplements.

The consortium of 7 universities, which initially formed NSBRI, was selected in 1997. Following this selection, several research protocols directed at countermeasure development against bone loss were competitively reviewed and four projects were selected to receive funding for the first three years of the program. This report reviews the accomplishments of the investigators involved in this funding cycle and will focus on how these contribute to the development of effective countermeasures against bone loss during extended spaceflight.

I. PROGRAM RESEARCH ACCOMPLISHMENTS

Program Synergy: Four research projects were initially approved for funding by NSBRI. Collectively the projects address the modulating effect 3 receptor agonists exert at the osteoblast and osteoclast level, specific mechanical factors (blood flow and mechanical loading) acting on bone cells to influence rates of bone resorption and the development of analytic methods, based on DXA, to assess bone mass and thus fracture risk in the femur. The questions initially posed by these projects may be summarized as follows:

1. Considering the essential role of hormone receptors in cell differentiation and function, what novel pharmacological agents can be developed to diminish osteoclastic bone resorption and to increase osteoblastic bone formation during, and after exposure to microgravity?
2. To what extent does decreased blood flow increase bone loss in the lower extremities during weightlessness?
3. What level of mechanical loading is required to maintain normal bone remodeling during microgravity conditions, and will pharmacologic intervention act synergistically with mechanical loading to limit bone loss?
4. How can net bone loss during microgravity be assessed in a timely manner, and what information does this provide about mechanisms facilitating bone loss and fracture risk?

Research Synergy

The projects reported here address the development of countermeasures from the cellular level (receptors) to the organ level (blood flow, vascular responses), to mechanical stimulation and antiresorptive therapy for the bone system, to the potential for real time assessment of changes in bone mineral density and bone structural changes. At each level, these investigations are interdependent as illustrated by the utility of measuring real time change in bone density applied to the effects of specific countermeasures tested during flight.

Research Accomplishments

Receptor Agonists: Three receptor agonist systems were studied: 1) estrogen receptor (ER) agonists of the SERM family because of the central role estrogen plays in the maintenance of bone mass in women and men, 2) vitamin D receptor (VDR) agonists were studied because of known alterations in vitamin D levels during spaceflight, and 3) a new class of receptor, the calcium sensing receptor (CaR) was studied because of the potential importance of this receptor in modulating bone cell differentiation and function. The aim was to determine if the separate and interactive response of bone cells would promote osteoblastic activity and decrease osteoclastic differentiation thus maintaining bone formation in excess of bone resorption. Both in vitro and in vivo studies have been accomplished.

Studies with the SERMs idoxifene and raloxifene have identified osteoblastic patterns of gene expression that are distinct for each of the agonists. A potentially effective countermeasure to vitamin D deficiency, the analogue EB 1089, which may also modulate cell differentiation has been defined. Multiple osteoblastic cell strains, have been shown to express the CaR opening the possibility for regulation of osteoblast/osteoclast differentiation using CaR receptor agonists. Interaction between receptor agonists has been investigated. Studies in the hindlimb suspended rat are in progress with the goal of in-flight testing. Thus, the overarching result of these studies rests not only in the definition of specific receptor responses to a series of agonists, but in the demonstration that the agonists interact with each of the individual receptors to promote osteoblastic activity and decrease osteoclastic activity. It is this synergy that will direct the future development of novel bone active agents.

Research Results (O'Malley et al):

1. CaR is expressed in most osteoblasts in sections of bovine, murine and rat bone. Osteoclastic cells and osteoclast precursor cells also express the CaR. These results support the role of calcium as a modulator of osteoblast/osteoclast differentiation and open the way for studies involving synergistic actions among these receptor agonists in both types of cells and their precursors.

2. CaR agonists are good candidates for increasing osteoblastic activity. CaR agonist NPS-R467 activates an outward K channel in osteoblastic cells, mediates chemotaxis of murine calvarial osteoblasts in response to elevated levels of Ca^{2+}.

3. EB 1089 is a more potent and effective vitamin D analogue compared to calcitriol in inducing artificial target genes or endogenous genes such as osteocalcin and alkaline phosphatase in MG-63 cells. This result suggests that Vitamin D receptor agonists such as EB 1089 may: 1) effectively substitute for endogenous D levels suppressed by flight, 2) avoid hypercalcemia and hypercalcuria, 3) suppress osteoclastic bone cell differentiation.

4. EB 1089 prevented loss of trabecular bone and cortical bone area in the hindlimb suspended rat. In contrast to calcitriol-treated animals, urine pyridinoline crosslink excretion is not increased in EB 1089 treated animals.

5. Studies with VDR agonists indicates that reciprocal changes occur in MAPK/Erk activation and VDR activity and the calcitriol-dependent activation of alkaline phosphatase.

6. Estradiol and SERMS (idoxifene and raloxifene) appear to induce distinct patterns of gene expression in ostoeblastic cells, suggesting that estradiol and SERMs regulated bone mass may occur via different mechanisms. ER mediated increased in vitamin D receptor expression support the hypothesis that combinations of ER and VDR--based countermeasures may be more effective than either agent alone.

7. Estradiol and the SERM raloxifene are being tested in 6 month old hindlimb suspended rats. Estradiol and Raloxifene maintain bone mass, particularly cortical bone, during hindlimb suspension. The VDR agonist EB 1089 may have a greater effect on preserving trabecular bone. Suggesting that combinations of EB 1089 and raloxifene may provide superior protection against unloading induced bone loss in comparison to either agent alone.

Bone Flow and Bone Resorption (Bloomfield et al): It is increasingly apparent that altered blood flow to the lower limbs and upper trunk during microgravity disturbs normal gravity-related fluid dynamics in bone, with correspondingly significant changes in cell function and the release of bone active mediators. The aims of this study included: 1) determination of whether unloading of hindlimb bone plus the cephalic fluid shift alter bone perfusion rates, 2) characterization of the time course of these responses, and 3) the effect of β-agonists in mitigating the decrease in blood flow due to unweighting. It is postulated that changes in transcortical and marrow blood flow decreases interstitial shear stress, and induces decreased osteoblastic PGE_2, PGI_2 and nitric oxide production and a reciprocal increase in osteoclastic activity leading to bone resorption. The genetic expression regulating these modulators is under study in the laboratories of L. Suva and R. Turner.

Research Results:

1. Hindlimb unloading, within 10 minutes, diminishes blood flow to the femoral and tibial methaphysis (trabecular bone), diaphysis (cortical bone) and marrow. Prolonged unloading, e.g., over 7 days in the hindlimb suspended preparation, further decreases perfusion of the femoral shaft and marrow. The prolonged decrease in blood flow to the marrow space is of interest in view of changes in marrow osteoeprogenitor cells after unloading.

2. The decline in blood flow to the hindlimb coincides with a diminished mineral apposition rate, diminished bone density and decreased mass of both cortical and trabecular bone. Correspondingly, increased blood flow and fluid shift cephalad coinides with reported increase in bone mass in the upper trunk and head following hindlimb suspension.

3. Gene expression patterns: In collaboration with Drs. Suva and Turner preliminary studies have demonstrated significant differences in gene expression following hindlimb suspension measured by gene array methods.

4. The countermeasure actions of β-agonist agents is currently under study. Determination of the time course of blood flow changes indicates that skeletal blood flow capacity during standing is diminished with 28 days of hindlimb standing: e.g., skeletal unloading alters the structure of osseous circulation. This raises the question of post-microgravity ischemia, and may also relate to the significant delay experienced in reconstituting normal bone mass after flight.

This study emphasizes the important role played by altered bone circulation during exposure to microgravity conditions. Studies of bone cell gene expression and cytokine production from marrow and vascular endothelial cells, now topics of great interest to the vascular physiologist finds ready application to the problem of bone loss. The definition of countermeasures for this problem requires additional investigation. Negative pressure lower body instrumentation may not provide the answer. Administration of various vasoactive agonist agents may also not be feasible because of the systemic effects of these agents. Nevertheless, maintenance of lower extremity blood flow is essential to the maintenance of bone/muscle health.

Effect of Graded Mechanical Loading and Ibandronate: in the Adult Rat Model of Microgravity (Schultheis et al)

Exercise programs employed during spaceflight over the past 30 years have failed to prevent either muscle or bone loss. As efforts are directed to 6 month flights in the ISS or possible 3 years exploration to Mars, there is increased emphasis on the development of effective countermeasures. Several laboratories have proposed mechanical systems to limit the detrimental effects of microgravity on the musculoskeletal system. These include resistive exercise, methods for providing the pull of artificial gravity in the spacecraft and mechanical loading systems. Of these, positive tissue responses have been reported after the application of mechanical strain induced by repetitive vibrational stimulation or impact stimulation of the lower extremities. Loading by partial weight bearing to simulate Mars gravity and added vibrational strain has been evaluated using a novel force plate with the hindlimb suspended rat. The effect of a simultaneously administered potent bisphosphonate, ibandronate (Roche), on bone density and other related parameters has been determined in this system.

Research Results

1. Using 3 mo old female rats, hindlimb suspension with 50% weight bearing on the forelimbs failed to maintain normal cortical or trabecular bone mass in the forelimb as compared with free-roaming controls. Partial weightbearing however, maintained bone formation rate and extrinsic and intrinsic (material) mechanical properties. Partial weightbearing preserved geometrical properties of cortical bone in 5 mo old animals.

2. Dynamic loading at 3 Hz which lies in the normal ambulatory spectrum of the rat superimposed on partial (50%) weightbearing was effective in preserving cortical bone area, polar moment of inertia and section modulus. Bone formation rate was maintained under these conditions. However, trabecular bone density and the stiffness of bone were not maintained.

3. Ibandronate treatment retarded the decrease in trabecular density and increased bone formation rate. Ibandronate increased trabecular bone density in free-roaming animals. Significant increases in structural properties of cortical bone were observed with ibandronate under conditions of partial weightbearing.

4. Biochemical analyses of bone matrix proteins in suspended animals demonstrates a decrease in collagen (ug/mg bone) and proteoglycan (ug/mg bone) compared to free-roaming controls, but no change in osteocalcin content. Ibandronate treatment increased collagen content but not proteoglycan in controls and suspended animals.

These results suggest that a combination of mechanical loading and pharmacologic inhibition of bone resorption with ibandronate, a potent third generation bisphosphonate, may provide optimal protection against bone loss in humans exposed to microgravity. Ibandronate may also have significant effects on matrix protein content.

Skeletal Structural Consequences Of Reduced Gravity Environments (Ruff et al.)

Protection against the risk of fracture while in microgravity environments for extended periods of time requires an understanding of the mechanisms involved in bone loss as well as the means to monitor sequential changes in bone mass. The primary goal of this project was to document changes in mechanically relevant geometric parameters of bone structure in humans and in animal models under microgravity conditions, during altered mechanical loading on the forelimb in hindlimb suspended rats, and during treatment with ibandronate, an antiresorptive bisphosphonate agent (see Schultheis). Three data sets were analyzed: 1) DXA data derived from pre- and post studies of Mir cosmonauts, 2) DXA data from human subjects at prolonged bedrest (Dr. Adrian LeBlanc, PI), and 3) pQCT measurements of bone mass parameters in rats subjected to 35 days of hindlimb suspension and treatment with ibandronate (Dr. L. Schultheis, PI). Changes in hip fracture risk due to prolonged spaceflight were estimated using data derived from a 3-D finite element analysis of the femur and a dynamic loading model.

Research Results:

1. 112 day bedrest leads to a decline in BMD and geometric section properties. Hip DXA analysis in control subjects before and after 17 weeks of bedrest shows a significant decline in femoral neck bending strength, as much as 2% /month. However, ranges of responses varied from an 8% decline to a 1% gain, highlighting not only the importance of consistency in experimental conditions, but also the wide variation in individual response to conditions of unloading. This variation in response, now demonstrated by geometric/structural analysis was also seen in BMD measurements following flight.

2. Hip DXA scans on Mir cosmonauts in collaboration with Dr. LeBlanc et al., studies after an average of 178 days in flight again showed a decline in bone section modulus of more than 1% per month in the femur neck. In contrast to structural changes associated with normal aging or in bed rest subjects, periosteal diameter did not increase at any proximal femur location. This absence of periosteal expansion in association with endocortical bone resorption thus increases fracture risk. Failure to increase the expression of genes related to local grwoth has been reported in the periosteal layer of rodents following flight by Turner et al.

3. 3-D finite element analysis based on DXA data derived from cadaveric specimens estimates fracture associated with falls after bone loss after 12 months of spaceflight. The initial factors of risk for fracture in a midstance loading configuration were 0.62 for males, and 0.71 for females representing an increase in factors of risk by 26%. This would apply to risk

following a traumatic event or that during work in a hazardous environment. Quantitatively similar results were obtained using 2-D curved beam theory analysis of DXA data. The Technology Team (Drs. Charles, Beck and Pisacane) could obtain such measurements during flight with the AMPDXA bone densitometer scanning instrument under construction at the Hopkins Applied Physics Laboratory.

4. pQCT measurements in the hindlimb-suspended rat model of microgravity (see Dr. Schuletheis' report for full data analysis) indicate that partial weight bearing conditions (50% forelimb weight bearing) may not suffice to maintain normal bone remodeling. Mechanical stimulation through forceplate vibration may maintain cortical, but not trabecular, bone mass. The administration of an antiresorptive bisphosphonate, ibandronate, does prevent bone loss under these conditions. The results suggest that combined mechanical loading plus a pharmacological intervention may be the optimal method for reducing bone loss under conditions of prolonged microgravity exposure.

II. RISK REDUCTION ACHIEVED BY PROGRAM

The research results presented above and in the Principal Investigators reports, are targeted at risk reduction through the development of specific countermeasures to bone loss. Effective mechanical loading which may maintain both muscle and bone mass is critical in this regard, 50% weight-bearing may not be effective in reducing bone loss. Mechanical stimulation by vibration reported here is also under study in other laboratories. Although optimal levels of vibrational or impact stimulation have yet to be defined, and the instrument by which this can be applied in the flight setting has yet to be designed, the results clearly point to vibrational systems as potentially important countermeasure in the effort to decrease bone loss.

An understanding of the relationship of bone loss to altered vascular responses during weightlessness is critical to effective countermeasure development. Current studied are aimed at the pharmacological and biomechanical methods for maintaining blood flow at a specific site in the lower trunk. Here, risk reduction depends on progress in this area.

Two classes of antiresorptive drugs have been studied in these projects, and on-going research is being pursued in the hind-limb suspended rat model. Bisphosphonate drugs clearly may be useful in mitigating bone loss associated with microgravity exposure experienced by humans. Correction of abnormal vitamin D metabolism due to microgravity appears possible through the use of newer vitamin receptor agonists. The results suggest that manipulation of the vitamin D receptor and estrogen receptor via the non-calcemic analog EB1089 and the bone selective ER agonist raloxifene appear to have potential in counteracting unloading-induced bone loss. As yet less defined, but approaching the level of animal experimentation are CaR agonists which probably will be effective in the regulation (positive and negative) of progenitor cell differentiation, both for osteoblasts and osteoclasts.

Furthermore, these reports extend the potential for a real-time assessment of fracture risk under conditions of prolonged spaceflight at 0 gravity and during Mars habitation at 0.38 X g. The studies reported here point to the necessity of developing a flight-compatible means for sequential measurements of bone density and bone structural properties during flight and during extraterrestrial habitation. As demonstrated in earth studies and in the Mir cosmonauts, the application of analyses reported by Dr. Ruff and his colleagues holds promise for the real-time assessment of countermeasures effectiveness. They afford the ability to modify activity levels, exercise programs or medication regimen to maintain optimal bone strength in each individual.

III. PROGRAMATIC IMPLICATIONS OF RESEARCH RESULTS

A major implication of these results is that the response of bone to microgravity probably involves mechanisms that are not fully explained by known alterations in traditional calcitropic hormones or mineral balance. Furthermore, the results suggest, pending additional research addressing the issue of mechanical loading, that *both* mechanical loading and pharmacological suppression of bone resorption will be important for the maintenance of bone mass during prolonged microgravity exposure.

Key issues standout as important foci for continued research:

1. The first NSBRI program did not permit addressing important critical path risks: fracture healing, soft tissue injury and renal stone formation. In addition, the interplay between muscle and bone has not been included in the projects initially funded. These topics will be addressed during the second three year cycle of funding.

2. The investigators on the Bone Team have gained a remarkable level of familiarity with elements of space physiology related to bone loss that are critical to the maintenance of a healthy crew during prolonged spaceflight. This expertise is at a point where closer working relationships with NASA scientists would enhance opportunities to move from ground to flight testing.

3. The results reported here emphasize the need to focus on countermeasure development. They illustrate the need to selectively encourage basic science, while at the same time, moving towards flight testing. This is evident in receptor agonist studies which have progressed from cellular investigations to animal testing, to the use of a novel intravenous bisphosphonate in rodents to testing these agents in humans in a chronic bed rest setting, and to definition of effective rates of vibrational stimulation pending the design of a flight-testable instrument. Similarly, collaborative investigation on bone structural characteristics and fracture risk can be continued in flight using a new and flight compatible bone density device under development by the Technology team.

RESEARCH AREA:	**Bone Loss**
PRINCIPAL INVESTIGATOR:	**Bert W. O'Malley, M.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Novel Receptor-Based Countermeasures to Microgravity-Induced Bone Loss**
FUNDING:	**$281,000 (FY 1998); $283,584 (FY 1999); $332,640 (FY 2000)**
TOTAL FUNDING:	**$897,224**

PROJECT EXECUTIVE SUMMARY

The biological actions mediated by the estrogen receptor (ER), vitamin D receptor (VDR) and extracellular Ca^{2+}_o-sensing receptor (CaR) play key roles in the normal control of bone growth and skeletal turnover that is necessary for skeletal health. These receptors act by controlling the differentiation and/or function of osteoblasts and osteoclasts, and other cell types within the bone and bone marrow microenvironment. The appropriate use of selective ER modulators (SERMs) which target bone, vitamin D analogs that favor bone formation over resorption, and CaR agonists that may both stimulate osteoblastogenesis and inhibit osteoclastogenesis as well as the function of mature osteoclasts, should make it possible to prevent the reduction in bone formation and increase in bone resorption that normally contribute to the bone loss induced by weightlessness. Indeed, there may be synergistic interactions among these receptors that enhance the actions of any one used alone. Therefore, we proposed to: 1) assess the *in vitro* ability of novel ER, VDR and CaR agonists, alone or in combination, to modulate osteoblastogenesis and mature osteoblast function under conditions of 1g and simulated microgravity; 2) assess the *in vitro* ability of novel ER, VDR and CaR agonists, alone or in combination, to modulate osteoclastogenesis and bone resorption under conditions of 1g and simulated microgravity; and 3) carry out baseline studies on the skeletal localization of the CaR in normal rat bone as well as the *in vivo* actions of our novel ER- and VDR-based therapeutics in the rat in preparation for their use, alone or in combination, in well-established ground-based models of microgravity and eventually in space flight.

We have found that the CaR is expressed in osteoblastic cells as well as in bovine, murine and rat bone and that activation of this receptor in osteoblastic cells leads to activation of an outward K^+ channel and chemotaxis of calvarial osteoblasts in response to elevated Ca^{2+}_o. The CaR is also present in osteoclast precursors and in osteoclast-like cells formed *in vitro* and plays roles in regulating osteoclastogenesis and osteoclast chemotaxis. In our VDR studies, we have examined the ability of the VDR agonist, EB1089, which is less calcemic than calcitriol, to regulate osteoblastic gene expression and find that it is more potent than calcitriol. In addition, gene expression in the MG-63 osteoblastic cell line was characterized in the Slow Turning Lateral Vessel (STLV) culture system, which approximates many aspects of microgravity. Many genes were down-regulated in comparison to monolayer cultures grown at unit gravity, and responses to VDR agonists were less robust. In ongoing hindlimb suspension studies in male rats, EB1089 was able to prevent unloading-induced bone loss measured at the proximal tibia, while calcitriol was able to increase bone mineral density. However, increases in serum calcium in calcitriol-treated animals, not observed in EB1089-treated rats, indicate that the latter is a superior countermeasure. EB1089 is also a less potent stimulator of osteoclast formation in comparison to calcitriol. Finally, our ER studies have revealed that osteoblastic gene expression patterns induced by estradiol and the SERMs idoxifene and raloxifene, are distinct even though all agents are capable of inhibiting bone loss due to sex steroid depletion. Raloxifene does not reduce bone

mineral density in normal female rats and has only modest effects on biochemical markers of bone turnover suggesting its use in gonad-intact populations should not increase the risk of bone loss via inhibiting endogenous estrogens. In ongoing hindlimb suspension studies in ovariectomized female rats, raloxifene and estradiol, individually, appear able to prevent loss of bone mineral density. Since raloxifene does not exert undesirable, estrogen-like effects in reproductive tissues, it has the potential to be an acceptable countermeasure to disuse-induced bone loss. Ongoing studies will continue to examine the use of EB1089 and raloxifene, alone and in combination, to prevent bone loss in male and female rats induced by hindlimb suspension.

Collectively these studies suggest that manipulation of VDR and ER activity has the potential to reduce the risk of bone loss resulting from the microgravity environment encountered during Space travel. Importantly, our data also suggest that novel ligands for these two receptors that significantly attenuate the negative side effects of the natural ligands can be effectively employed to reduce unloading-induced bone loss, and the ensuing risks of bone fracture.

RESEARCH AREA:	**Bone Loss**
PRINCIPAL INVESTIGATOR:	**Susan A. Bloomfield, Ph.D.**
ORGANIZATION:	**Texas A&M University**
PROJECT TITLE:	**Bone Blood Flow During Simulated Microgravity: Physiological and Molecular Mechanisms**
FUNDING:	**$187,478 (FY 1998); $217,217 (FY 1999); $246,374 (FY 2000)**
TOTAL FUNDING:	**$651,069**

PROJECT EXECUTIVE SUMMARY

Blood flow to bone has been shown to affect bone mass and presumably bone strength. Preliminary data indicate that blood flow to the rat femur decreases after 14 days of simulated microgravity, using hindlimb unloading (HU). If adult rats subjected to HU are given dobutamine, a synthetic catecholamine which can cause peripheral vasodilation and increased blood flow, the loss of cortical bone area usually observed is prevented. The primary aim of this project is to characterize changes in 1) bone blood flow, 2) indices of bone mass, geometry, and strength, and 3) changes in gene expression for candidate genes for mechanotransduction in bone after 3, 7, 14, 21, and 28 days of HU in the adult rat. Using a rat of at least 5 months of age avoids inadvertently studying effects of simulated microgravity on growing, rather than adult, bone.

The key contributions of these studies lie in the quantification of blood flow to various compartments of bone and marrow with the acute adjustment to the tail suspension posture (after 10 minutes) and after 7 and 28 days of tail suspension. Ten minutes of HU significantly decreases flow to the proximal femur, the femoral shaft and to femoral shaft marrow. By 7 days of HU, blood flow to all portions of the femur, including distal metaphysis, is sigificantly less than flow to these regions in cage-activity control rats. These decreases in flow are due to an increase in vascular resistance; this increased resistance is maintained through 28 days of HU. Conversely, blood flow to bone of the forelimb and head (skull, mandible) is increased during HU with the acute shift to the tail suspension posture. However, blood flow returns to normal with 7 days HU due to compensatory increases in vascular resistance. These alterations in blood flow were accompanied by changes in bone mass, with those bones experiencing lower flows during HU decreasing in mass (femur, tibia), whereas those bones experiencing that acute increase in flow with HU increasing in mass (mandible, clavicle, humerus).

Because radiolabeled microspheres are used to measure blood flow, rendering all tissues radioactive, bones from these animals could not be further processed. Separate experiments were performed to document the time course of alterations in bone mineral content, bone geometry, mechanical properties and gene expression with HU. Histomorphometric analyses of fluorochrome-labeled bone reveal large decrements in mineral apposition rate and bone formation rate at the tibio-fibular junction (60% and 90% declines, respectively) in these mature adult rats. Our data confirm that of earlier investigators (Dehority et al., *Amer. J. Physiol.*, 1999) in that decreases in bone formation are slower to develop in the mature rat skeleton as opposed to the better characterized response of young growing rats, but more prolonged.

Few changes were noted in bone mineral density (BMD) of mid-shaft cortical bone. Alterations in tibial BMD and cross-sectional area appear to parallel growth-related changes in the humerus. Not surprisingly, mechanical properties at this site were not affected either. Analyses using

pQCT of the proximal tibial metaphysis reveal a compartment-specific alterations in BMD, where cancellous (trabecular) bone is decreasing in BMD even as the cortical shell gains in BMD during 28d HU. Novel analyses of cancellous bone mechanical properties reveal a decline in both elastic modulus and ultimate stress in this core of the proximal tibial metaphysis after 28d HU. Clearly, mechanical strength of bone is compromised at sites rich in cancellous bone in the unloaded limb, even in the mature adult skeleton in which growth processes are minimal.

Gene expression is altered in proximal femur samples from these unloaded rats. Early changes at 3 days reveal an increased expression of cytokines favoring bone resorption (interleukin-6 and interferon-γ) and a decrease in expression of TGF-β1, which normally stimulates bone formation activity. These alterations remain to be confirmed in longer duration studies. Microarray data indicate upregulation of a number of genes potentially important in regulating bone cell activity: integrin abv3 and abv5, nitric oxide synthase, prostaglandin synthase 1 and 2. A down-regulation of several oncogenes (fos, abl) was noted, as well as for BMP receptors types I and II. A very recent finding with micro-array analysis points to an upregulation of a membrane kinase previously unidentified in bone in proximal femurs from rats subjected to 28d HU.

These results have important implications for the development of countermeasures to ameliorate bone loss with prolonged exposure to weightlessness. We have established a time course for declines in blood flow to bone, using state-of-the-art microsphere studies, which precede reductions in bone formation (at mid-shaft tibia) and BMD and mechanical propertiesof cancellous bone in the proximal tibia. If, indeed, continuing experiments can demonstrate a causal link between altered blood flow and shifts in bone remodeling activity, a whole new category of countermeasures might be considered. Some pharmacological agents may effect a general vasodilation and therefore increase in blood flow, but relatively benign physical measures [heating, exercise, lower body negative pressure (with or without exercise)] can be tested for effectiveness in earth-based studies in increasing blood flow to bone. These data on mature adult rats, a better model for adult human bone than the more widely used growing rat, also suggest that sites rich in cancellous bone (proximal femur, proximal tibia, distal femur) in unloaded limbs are at the highest risk for fracture with prolonged exposure to microgravity. We may have over-estimated the rate of change in cortical bone BMD and cortical bone geometry in the past having relied on information from rapidly growing young rats. Our gene expression data provide some mechanistic data on which to build future countermeasure strategies, given early changes in regulatory peptides important in regulating bone cell activity.

RESEARCH AREA:	**Bone Loss**
PRINCIPAL INVESTIGATOR:	**Lester W. Schultheis, M.D. (Replacing J. R. Shapiro)**
ORGANIZATION:	**Johns Hopkins School of Medicine**
PROJECT TITLE:	**The Effects of Partial Mechanical Loading and Ibandronate on Skeletal Tissues in the Adult Rat Hindquarter Suspension Model for Microgravity**
FUNDING:	**$279,000 (FY 1998); $242,939 (FY 1999); $321,970 (FY 2000)**
TOTAL FUNDING:	**$843,909**

PROJECT EXECUTIVE SUMMARY

The loss of bone mass as a result of weightlessness assumes increasing importance as the duration of manned spaceflight increases. To date, efforts to maintain skeletal mass using a variety of exercise techniques or dietary modifications have proven less than effective. Our central hypothesis was that pharmacological modification of bone resorption combined with precisely designed mechanical loadbearing, may provide the best possible skeletal protection.

In earlier work, our laboratory rats were placed in a harness and counterweight system that lifted a known fraction of weight off their legs. These data constituted a preliminary dose-response relationship between weightbearing and bone. In our work with NSBRI, we focused on the 50% weightbearing condition to examine the effect of partial weightbearing because it provides a benchmark to estimate the effect of Martian gravity and artificial gravity on bone. Based upon evidence that osteogenesis is particularly sensitive to specific components of dynamic bone strain, we developed a unique instrument to control mechanical loadbearing on the front limbs of hindquarter suspended rats. This consisted of a platform controlled by a digital computer in negative feedback so that it would resonate with specified frequencies of impact. Our system enabled us to process aperiodic forces in the frequency domain as they are applied to bone through normal joint contact and muscle insertions during a form of ambulation. We maintained the normal spectral (Fourier component) composition of quadrupedal forces in a bipedal rat. Partial weightbearing was controlled as a simulation of reduced gravity independently from dynamic forces that simulated carefully designed exercise. We suspected that simultaneous pharmacologic inhibition of resorption and bone turn-over would be more effective with mechanical countermeasures than either regimen alone. We hoped to provide an accelerated model for the NASA JSC human bedrest studies of LeBlanc and Shackleford testing the effects of episodic resistive exercise and bisphosphonate therapy because of the more rapid bone turnover in the rat. The endpoints of the proposed treatments will include biochemical, cellular, histological, mechanical, and gross anatomical skeletal analysis in each animal and an assessment of systemic stress. No prior studies have include as completed a range of analytical procedures in the same animal.

RESEARCH AREA:	**Bone Loss**
PRINCIPAL INVESTIGATOR:	**Christopher B. Ruff, Ph.D.**
ORGANIZATION:	**Johns Hopkins University**
PROJECT TITLE:	**Skeletal Structural Consequences of Reduced Gravity Environments**
FUNDING:	**$168,000 (FY 1998); $172,031 (FY 1999); $172,031 (FY 2000)**
TOTAL FUNDING:	**$512,062**

PROJECT EXECUTIVE SUMMARY

Bone loss under conditions of microgravity has been widely recognized as a potentially critical obstacle to carrying out long-term space missions. Loss of bone mass and density during spaceflight has been documented in both experimental animals and human astronauts and cosmonauts for several decades. However, the structural consequences of such bone loss, in terms of changes in bone strength and increased fracture risk, are much less well understood. Bone strength is a function of both the amount of material present (i.e., mass) and its geometric distribution (i.e., structure). The primary goal of this study was to document changes in mechanically-relevant parameters of bone structure in humans and animal models under conditions of microgravity, and to assess the effects of applied mechanical and pharmacological countermeasures on these changes. Using data derived from a detailed structural analysis of the proximal femur and a dynamic loading model, changes in hip fracture risk after prolonged spaceflight were estimated. The effectiveness of three potential countermeasures – partial weight-bearing, administration of a bisphosphonate, and vibrational mechanical stimuli – on bone strength were evaluated using a tail-suspended rat model. We have thus made major strides towards addressing the goals put forth in the NSBRI Critical Research Path and Bone Team objectives: to assess the probability of a fracture occurence without countermeasures, and to assess the efficacy of different countermeasures in preventing fractures.

The project had three complementary aims: 1) to measure bone structural changes in the hip, extracted from 2-D DXA (dual-energy x-ray absorptiometric) data, in humans subjected to microgravitational conditions, including bedrest on Earth and living aboard the Mir Space Station; 2) to construct 3-D finite element models of the hip from cadaveric femoral specimens which could then be altered using results from (1) to simulate microgravitational effects, and used together with dynamic link analysis to calculate changes in fracture risk; and 3) to assess the effectiveness of several countermeasures - partial (.5 G) mechanical loading, the administration of a bisphosphonate (ibandronate), and mechanical vibrational stimulation - on bone structural changes using a tail-suspended rat model. All of these objectives were accomplished during the three years of support.

Both the bedrest and Mir subjects showed significant declines in measures of bone strength during exposure to microgravity. Declines in the section modulus, an index of bending strength, were comparable in the proximal femoral shaft in bedrest and Mir subjects, averaging almost 1%/month, but rates of decline in the same index in the femoral neck averaged about twice as large in the Mir subjects (1.3%/month) than in the bedrest subjects (.7%/month), despite similar rates of decline in BMD (bone mineral density) (1.3 and 1.15%, respectively). The difference in section modulus changes in the femoral neck was due largely to a small but significant increase in the outer (periosteal) diameter of the bone in the bedrest subjects, an effect not seen in the Mir group. Follow-up studies of the Mir cosmonauts after return to Earth showed an increase in

periosteal diameter of the femoral neck which helped to restore its strength, similar to some age changes that we have observed in the normal elderly population. We interpret these findings to indicate that a) geometry, not just bone mineral mass or density, is important in assessing bone strength, b) patterns of change in bone structure in spaceflight subjects are in some ways unique, and thus c) extrapolations from Earth-based studies may be misleading, and furthermore d) detailed geometric measurements should be included in any bone monitoring protocol during spaceflight and/or Mars exploration.

Two 3-D finite element (FE) models of the proximal femur were constructed from cadaveric specimens from a 36 year-old male and 32 year-old female. 3-D FE analysis allows a much more detailed and realistic modeling of both the geometry and loading conditions of the hip region than is possible using 2-D DXA-derived measurements. The failure loads and risk of fracture following a fall to the side (assuming an average body height and weight) were calculated for each femur, using a dynamic link model that accurately reflects in-vivo loadings. Using the structural information available from the DXA cosmonaut data and some assumptions derived from other experimental studies, these 3-D models were then altered to reflect the average change in structure that would occur after a year of spaceflight, and failure loads and fracture risks were recalculated. Load to failure was reduced by more than 20% on average in the two femora, resulting in an increase in risk for fracture averaging almost 30%. Because we found significant individual variation in how much bone is lost, and how much strength is reduced following exposure to microgravity in both the Mir and bedrest subjects, it is very likely that changes in failure load and fracture risk in some individuals would be even more extreme than these mean estimates. We also compared these results to those obtained through a simpler 2-D curved beam analysis, and found that predicted failure loads were comparable using the two models. The 2-D curved beam analysis has the advantage that it could be applied directly to DXA data gathered during spaceflight, thus enabling longitudinal monitoring of bone changes in astronauts/cosmonauts if a DXA-like apparatus were included on board.

In the tail-suspended rat study, rats were subjected to 35-day periods of partial weight-bearing using a custom-designed platform and mechanical feedback device. Long bone structural parameters were measured before and after treatment using pQCT (peripheral quantitative CT). Results of these studies indicated the following: 1) .5 G loading (similar to the Martian environment) is not sufficient to maintain bone strength. 2) Administration of ibandronate is an effective countermeasure for loss of bone strength under microgravitational conditions. 3) Mechanical stimulation via vibrations applied to the supporting substrate is also an effective countermeasure for maintenance of cortical bone strength, although not trabecular bone density. Thus, a combination of both pharmacologic and mechanical treatments may be necessary to maintain bone strength under microgravitational conditions.

These results show that bone distribution, or structure is a major factor in determining strength and fracture risk. This has implications not only for planning as part of the Critical Research Path for Mars exploration, but also for Earth-based health applications, in particular age-related osteoporosis. Fracture risk is a major medical problem among the elderly. Consideration of all structural components of a skeletal element should improve fracture risk evaluation; in fact, we are currently engaged in parallel studies of bone structural changes in several large demographic samples of the normal population, including the NHANES national survey and the SOF (Study of Osteoporotic Fractures). Studies of these kinds, carried out from a mechanical perspective, should aid in our understanding of both the etiology and consequences of bone loss under a variety of environmental conditions, and provide more accurate evaluation of the efficacy of countermeasures.

NSBRI RESEARCH PROGRAM
CARDIOVASCULAR ALTERATIONS

Team Leader: Cohen, R. J. MIT

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CARDIOVASCULAR ALTERATIONS TEAM PROGRAM EXECUTIVE SUMMARY

The cardiovascular system seems to function remarkably well during conditions of space flight. However, particularly during prolonged space flight, the process of cardiovascular deconditioning impairs the cardiovascular system's ability to readapt to a gravity environment. Upon reentry from space flight into the Earth's gravitational field, astronauts experience orthostatic hypotension and reduced exercise capacity, which limits their ability to function during reentry and after landing. For example, in many cases, the orthostatic hypotension is sufficiently severe that astronauts cannot stand erect for some time after landing, thus precluding emergency egress on Earth or another planetary surface. Despite years of research, the mechanisms leading to orthostatic intolerance following microgravity exposure remain poorly characterized and current countermeasures are not adequately effective.

One aspect of cardiovascular deconditioning is a reduction in cardiac mass, the mechanism of which is not known, nor are its functional correlates and reversibility known. In addition, there is significant anecdotal evidence that space flight is associated with decreased cardiac electrical stability which may pose a life threatening risk to astronauts. For example, one crew member during the Skylab missions had a five beat run of ventricular tachycardia during lower body negative pressure. Furthermore, analysis of nine 24 hour Holter monitor recordings obtained during long term space flight on Mir revealed one 14 beat run of ventricular tachycardia. Possible mechanisms of arrhythmias and countermeasure strategies have barely been addressed. As long duration missions and older astronauts become more common, alterations in cardiovascular function resulting space flight are more likely to have an impact on mission success and astronaut safety. Thus, it becomes imperative to understand mechanisms of cardiovascular deconditioning and to develop appropriate countermeasures. The death of an experimental primate shortly after return from space, with cardiovascular mechanisms suspected as primary or contributing causes, lends urgency to these objectives.

The objective of this research program is to apply the most powerful technologies available to determine, in ground-based studies, the mechanisms by which space flight affects cardiovascular function, and then on the basis of an understanding of these mechanisms to develop rational and specific countermeasures.

The research effort is divided among seven projects:

1. Human Studies Core – Gordon H. Williams, PI
   This project revolves around a 16 day head down tilt bed-rest study that examines the mechanisms by which bed-rest and disruption of circadian rhythms affect cardiovascular hemodynamic regulation and cardiac electrical stability.

2. Alterations in Cardiovascular Regulation and Function During Simulated Microgravity – Richard J. Cohen, PI
   This study involves the application of a number of powerful new non-invasive measurement technologies, including cardiovascular system identification (CSI) for the assessment of closed-loop cardiovascular regulation in order to understand mechanisms involved in the development of orthostatic intolerance during microgravity simulated by bed-rest and to develop effective countermeasures.

3. Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity – Gordon H. Williams, PI
   This project studies alterations in the responsivenes of the renin-angiotensin-aldosterone hormonal salt and fluid regulatory system in response to the head down tilt bed-rest model of weightlessness. The goal of the project is to understand how these systems lead to the development of orthostatic hypotension and to develop effective countermeasures.

4. Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias During Simulated Microgravity – Richard J. Cohen, PI
   This study involves measurement of microvolt level T wave alternans measure of before and after bed-rest in order to determine whether simulated microgravity alters cardiac electrical stability.

5. Cardiovascular Deconditioning in Rodents – Artin A. Shoukas, PI
   Mechanisms involved in the development of orthostatic intolerance are studied in the tail suspended rodent model while taking advantage of the more invasive measurements that can be made in the rodent model as compared to the human model. The rodent model may also serve as a platform to test potential countermeasures before they are evaluated in human studies.

6. Computational Models of the Cardiovascular System – Roger Kamm, PI
   In this project, a computer model that simulates the critical components and behaviors of the cardiovascular system is being developed. The goal of this project is to understand how microgravity alters cardiovascular function and leads to the development of orthostatic hypotension. This model is validated using the data collected in the rodent and human studies and used to test potential countermeasures.

7. Cardiac Atrophy – Michael Schneider, PI
   The objective of this project is to determine the cellular and genetic mechanisms by which cardiac mass is reduced during space flight and to develop appropriate countermeasures using a unique rodent model of cardiac unloading.

These studies address the major cardiovascular problems associated with space flight. The plan, in each case, is first to determine the basic mechanisms of the cardiovascular alterations and then, on the basis of the understanding of these mechanisms, to propose and test rational, specific countermeasures. For the current year these studies are mandated to involve only ground-based studies, but as described below, we plan to develop proposals for flight experiments as well.

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Gordon Williams, M.D.**
ORGANIZATION:	**Brigham and Women's Hospital**
PROJECT TITLE:	**Cardiovascular Deconditioning in Humans: Human Studies Core**
FUNDING:	**$420,000 (FY 1998); $430,080 (FY 1999); $390,916 (FY 2000)**
TOTAL FUNDING:	**$1,240,996**

PROJECT EXECUTIVE SUMMARY

Major cardiovascular problems, secondary to cardiovascular deconditioning, may occur on extended space missions. While it is generally assumed that the microgravity state is the primary cause of cardiovascular deconditioning, sleep deprivation and disruption of diurnal rhythms may also play an important role. Factors that could be modified by either or both of these perturbations include: autonomic function and short-term cardiovascular reflexes, vasoreactivity, circadian rhythm of cardiovascular hormones (specifically the renin-angiotensin system) and renal sodium handling and hormonal influences on that process, venous compliance, cardiac mass, and cardiac conduction processes. The purpose of the Human Studies Core is to provide the infrastructure to conduct human experiments that allow the assessment of the likely role of such factors in the space travel associated cardiovascular deconditioning process, and to develop appropriate countermeasures. The Core takes advantage of the General Clinical Research Center at the Brigham and Women's Hospital, Boston, Massachusetts, to perform these studies.

The Core includes two general experimental protocols. The first protocol involves a head down tilt bed-rest study to simulate microgravity. The second protocol includes the addition of sleep deprivation to the simulated microgravity environment. Before and after each of these environmental manipulations, the subjects undergo acute stressors simulating changes in volume and/or stress, which could occur in space and on return to Earth. The subjects are maintained in a rigidly controlled environment with fixed sleep cycles, activity pattern, and dietary intake of nutrients, fluids, ions and calories. Within the Core experimental protocol framework, investigators perform specific experiments, some based on the application of new non-invasive measurement techniques, to determine the effect of the environmental modifications on the status and responsiveness of the cardiovascular, endocrine, and renal homeostatic systems. In the project led by Professor Cohen, titled Alterations in Cardiovascular Regulation and Function during Simulated Microgravity, investigators apply cardiovascular system identification (CSI) techniques to characterize important cardiovascular regulatory responses including the heart rate and peripheral resistance baroreflexes. The application of CSI involves the use of echocardiography for continuous beat to beat measurement of stroke volume. In the project led by Professor Williams, titled Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity, investigators characterize the renal and endocrine responses. Finally, in second project led by Professor Cohen, titled Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated Microgravity, investigators apply novel techniques to quantify changes in the cardiac conduction processes and assess any increased tendency toward cardiac arrhythmias or cardiac electrical alterations. Together, these projects cover a broad spectrum of systems involved in maintaining cardiovascular homeostasis and promise to provide new insight regarding their alterations in response to the major environmental changes of microgravity and disruption of circadian rhythms. The data from these studies is being used to develop and test potential countermeasures so as to ensure the health, productivity, and safety of astronauts during and on return from extended missions.

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Richard Cohen, M.D., Ph.D.**
ORGANIZATION:	**Massachusetts Institute of Technology**
PROJECT TITLE:	**Cardiovascular Deconditioning in Humans: Alteration in Cardiovascular Regulation and Function during Simulated Microgravity**
FUNDING:	**$140,000 (FY 1998); $130,860 (FY 1999); $182,524 (FY 2000)**
TOTAL FUNDING:	**$453,384**

PROJECT EXECUTIVE SUMMARY

Alterations in cardiovascular regulation and function that occur during and after space flight have been reported. These alterations are manifested, for example, by reduced orthostatic tolerance upon reentry to the earth's gravity from space. However, the precise physiologic mechanisms responsible for these alterations remain to be fully elucidated. Perhaps, as a result, effective countermeasures have yet to be developed. In this project we applied a powerful, new method – cardiovascular system identification (CSI) – for the study of the effects of space flight on the cardiovascular system in order to develop effective countermeasures.

CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate, arterial blood pressure (ABP), and instantaneous lung volume (ILV – respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of all the physiologic mechanisms coupling these signals, CSI provides a model of the closed-loop cardiovascular regulatory state in an individual subject. The model includes quantitative descriptions of the heart rate baroreflex, autonomic function, as well as other important physiologic mechanisms.

We applied CSI in conjunction with the bed rest protocol of the Human Studies Core project. This protocol involves ground-based, human head down tilt bed rest to simulate microgravity and acute stressors – upright tilt, standing and bicycle exercise – to provide orthostatic and exercise challenges. We found that a number of autonomically mediated responses, in particular the heart rate baroreflex, were diminished as a result of head down tilt bed rest.

Based on review of our preliminary human data, as well as based on animal data and computer simulation data obtained by other investigators in the NSBRI Cardiovascular Alterations Team, we decided to test a pharmacologic countermeasure which is applied at the very end of the bed rest period. This countermeasure was applied using the same bed rest protocol used to obtain the control data. This double blinded prospective evaluation of the countermeasure demonstrated that it was successful in diminishing orthostatic intolerance.

This project has led to better understanding of the mechanisms of orthostatic intolerance and the identification of a potential pharmacologic countermeasure. The CSI methodology used in this study can be also applied to the study of patients with a range of diseases that alter closed loop cardiovascular regulation such as heart failure, diabetes, and hypertension and may also be used to monitor treatment of these patients.

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Gordon H. Williams, M.D.**
ORGANIZATION:	**Brigham and Women's Hospital**
PROJECT TITLE:	**Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity**
FUNDING:	**Included in Human Studies Core Funding**

PROJECT EXECUTIVE SUMMARY

The volume regulating systems are integrated to produce an appropriate response to both acute and chronic volume changes. Their responses include changing the levels of the hormones and neural inputs of the involved systems and/or changing the responsiveness of their target tissues. Weightlessness during space travel produces a volume challenge that is unfamiliar to the organism. Thus, it is likely that these volume regulatory mechanisms may respond inappropriately, *e.g.*, a decrease in total body volume in space and abnormal responses to upright posture and stress on return to Earth. A similar "inappropriateness" also can occur in disease states, *e.g.*, congestive heart failure. While it is clear that weightlessness produces profound changes in sodium and volume homeostasis, the mechanisms responsible for these changes are incompletely understood. Confounding this analysis is sleep deprivation, common in space travel, which can also modify volume homeostatic mechanisms.

The purpose of this project is to provide the required understanding and then to design appropriate countermeasures to reduce or eliminate the adverse effects of microgravity. To accomplish this we are addressing five Specific Aims: 1) To test the hypothesis that microgravity modifies the acute responsiveness of the renin-angiotensin-aldosterone system (RAAS) and renal blood flow; 2) Does simulated microgravity change the circadian rhythm of the volume-regulating hormones?; 3) Does simulated microgravity change the target tissue responsiveness to angiotensin II (AngII)?; 4) Does chronic sleep deprivation modify the circadian rhythm of the RAAS and change the acute responsiveness of this system to posture beyond what a microgravity environment alone does?

Because the renin-angiotensin system (RAS) plays a pivotal role in blood pressure control and volume homeostasis, it likely is a major mediator of the adaptive cardio-renal responses observed during space missions and will be a special focus of this project. Thus, the overall goal of this project is to assess the impact of microgravity and sleep deprivation in humans on volume-regulating systems. To achieve this overall objective, we are evaluating renal blood flow and the status and responsiveness of the volume-regulating systems (RAAS, atrial natriuretic peptide and vasopressin), and the adrenergic system (plasma and urine catecholamines) in both simulated microgravity and normal gravity with and without sleep deprivation. Furthermore, the responses of the volume homeostatic mechanisms to acute stimulation by upright tilt testing, standing and exercise are being evaluated before and after achieving equilibrium with these interventions.

This work has implications for the treatment and prevention of maladaptive hemodynamic responses experienced by astronauts in flight and on return to Earth. It will increase our understanding of the mechanisms by which weightlessness and sleep deprivation change plasma volume and sodium homeostasis, thereby, providing entree to develop appropriate countermeasures.

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Artin A. Shoukas, Ph.D.**
ORGANIZATION:	**Johns Hopkins University School of Medicine**
PROJECT TITLE:	**Rodent Studies of Cardiovascular Deconditioning**
FUNDING:	**$175,000 (FY 1998); $204,199 (FY 1999); $214,199 (FY 2000)**
TOTAL FUNDING:	**$593,398**

PROJECT EXECUTIVE SUMMARY

Changes in blood pressure can occur for two reasons: 1) A decrease in cardiac output resulting from the altered contractility of the heart or through changes in venous filling pressure via the Frank Starling mechanism or; 2) A change in systemic vascular resistance. The observed changes in cardiac output and blood pressure after long term long term space flight cannot be entirely explained through changes in contractility or heart rate alone. Therefore, alterations in filling pressure mediated through changes in systemic venous capacitance and arterial resistance function may be important determinants of cardiac output and blood pressure after long term space flight. Our laboratory has previously shown the importance of veno-constriction mediated by the carotid sinus baroreceptor reflex system on overall circulatory homeostasis and in the regulation of cardiac output.

Summary of Accomplishments:
Our proposed experiments test the overall hypothesis that alterations in venous capacitance function and arterial resistance by the carotid sinus baroreceptor reflex system are an important determinant of the cardiac output and blood pressure response seen in astronauts after returning to earth from long term exposure to micro-gravity. This hypothesis is important to our overall understanding of circulatory adjustments made during long term space flight. It also provides a framework for investigating counter measures to reduce the incidence of orthostatic hypotension caused by an attenuation of cardiac output. We continue to use hind limb unweighted (HLU) rat model to simulate the pathophysiological effects as they relate to cardiovascular deconditioning in micro-gravity. We have used this model to address the hypothesis that micro-gravity induced cardiovascular deconditioning results in impaired vascular responses and that these impaired vascular responses result from abnormal alpha-1 AR signaling. The impaired vascular reactivity results in attenuated blood pressure and cardiac output responses to an orthostatic challenge.

We have used in vitro vascular reactivity assays to explore abnormalities in vascular responses in vessels from HLU animals and, cardiac output (CO), blood pressure (BP) and heart rate (HR) measurements to characterize changes in hemodynamics following HLU. Overall, we have been able to show that micro-gravity exposure is associated with a decrease in sympathetic neurotransmission (SN). This in turn is associated with a decrease in alpha-1 AR number and signaling as well as vessel smooth muscle mass (trophic effects of NE). Upon return to gravity, attenuated vascular contractility occurs secondary to end organ hyporesponsivenss, despite normal or accentuated sympathetic neurotransmission. Impaired venular and arteriolar responses to catecholamine stimulation results in impaired stroke volume, cardiac output and blood pressure responses. Specifically,

1. We have demonstrated impaired CO responses to an orthostatic challenge in rats following HLU which recovers in ~60hrs. (attached manuscript: submitted, Am J Physiol)
2. We have demonstrated that after HLU, unstressed venous vascular volume is increased following HLU and can no longer decrease in response to sympathetic stimulation. This

supports our primary hypothesis and may underlie the mechanisms leading to an exaggerated fall in stroke volume seen in astronauts. (attached manuscript in press, J Appl Physiol)

3. Using cardiopulmonary bypass studies in which cardiac output is fixed, we have demonstrated that venous an total circulatory capacitance is increased following HLU. (attached manuscript in press, J Appl. Physiol)
4. We have demonstrated impaired alpha-1-AR and non–alpha mediated responses in large arteries (aorta) of HLU animals. We have also demonstrated that the observed vascular contractile hyporesponsiveness is reversible with time, In addition, alpha-1AR specific abnormalities in mesenteric microvessel responsiveness appear to be present. (attached manuscript in review, J Appl. Physiol)
5. We have observed a decrease in alpha-1AR specific radioligand binding in aortic vessels from HLU animals.
6. We have demonstrated both an endothelial dependent and endothelial independent component which contributes to vascular hyporesponsiveness following HLU.
7. We have demonstrated vascular hyporesponsiveness in the large pulmonary arteries of the HLU rats. This vascular hyporesponsiveness is obliterated with nitric oxide synthase inhibition suggesting that increased nitric oxide production may be mediating this impaired contractile response.
8. We have demonstrated an impaired heart rat and blood pressure response to a orthostatic stimulus (transient bilateral carotid occlusion) in a HLU mouse model.
9. We have developed an external non-invasive mechanical prototype device, in conjunction with the Applied Physics Laboratory of JHU, that peristaltically pumps blood from lower extremities and abdomen towards the heart to maintain stroke volume and cardiac output during an orthostatic challenges. A notice of invention and non-disclosure has been filed with Johns Hopkins University.

In general our data support the hypothesis that vascular and specifically venular hyporesponsiveness is likely to contribute to impaired stroke volume response and blood pressure regulation following microgravity. We have also continued to validate the HLU rat model as a model that recapitulates the cardiovascular changes that occur following microgravity in humans. These accomplishments have allowed us to refine mechanisms, begin to test countermeasures, and bridge the gap between animal models and human subjects in our understanding of micro gravity induced orthostatic intolerance.

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Roger D. Kamm, Ph.D.**
ORGANIZATION:	**Massachusetts Institute of Technology**
PROJECT TITLE:	**Computational Models of the Cardiovascular System and its Response to Microgravity**
FUNDING:	**$75,000 (FY 1998); $76,800 (FY 1999); $76,800 (FY 2000)**
TOTAL FUNDING:	**$228,600**

PROJECT EXECUTIVE SUMMARY

Computational models of the cardiovascular system are powerful adjuncts to ground-based and in-flight experiments. They provide a rational framework that quantitatively defines interactions among complex cardiovascular parameters, supports the critical interpretation of experimental results and testing of hypotheses, and permits prediction of the impact of specific countermeasures.

Over the past 3 years we have implemented a computational model of the cardiovascular system capable of simulating the short term (0-5 min), transient response to orthostatic stress tests such as tilt/standing and lower body negative pressure (LBNP) in normals and microgravity adapted individuals. The model consists of a lumped parameter representation of the hemodynamic system and set-point representations of the cardiopulmonary and the arterial baroreflexes. The model allows for regional blood pooling in four systemic circulatory branches and three central venous compartments. Furthermore, we implemented non-linear venous pressure-volume characteristics for all dependent venous vascular compartments and allowed blood volume to change as a function of time and orthostatic stress to simulate blood plasma sequestration into the interstitium during orthostatic stress. We accounted for a reduction in the hydrostatic pressure component at the carotid sinus during tilt by making the input pressure to the arterial baroreflex a function of orientation in the gravitational field.

We verified the model under baseline (supine) conditions and after three minutes of orthostatic stress by comparing the model's predictions to a limited set of population-averaged data found in the medical literature. We also studied the transient response of the cardiovascular system to sudden gravitational stress. The experimental steady state and transient responses are well matched by the simulator.

By appropriately modifying some of the model's parameters we systematically simulated a number of proposed hypotheses of the mechanisms underlying post-flight orthostatic intolerance. The modeled hypotheses included hypovolemia, cardiac atrophy, increased leg venous compliance, decreased gain of the heart rate baroreflex, and a reduced ability to constrict venous and arterial smooth muscle. By simulating a tilt test response under these altered baseline conditions, we were able to compare the simulator's predictions to astronaut stand test data post-spaceflight. Our simulations indicate that although hypovolemia is the biggest single contributor, no single mechanism can account for the altered post-spaceflight heart rate dynamics. Rather, our simulations suggest that a superposition of reduced vasoconstriction of arterial and venous smooth muscle and hypovolemia can account for the dynamics of the heart rate response seen in astronauts post-flight.

The computational model was subsequently used to simulate the effects of a potential pharmacologic countermeasure, midodrine: an alpha agonist. We simulated the action of midodrine on a spaceflight adapted individual by increasing total peripheral resistance and decreasing unstressed venous volume by amounts compatible with experimental findings. The simulated post-flight heart rate response of a midodrine-treated individual demonstrates a significant reduction in the excessive tachycardia. The simulation suggests that the drug may have major potential benefit as a countermeasure for orthostatic intolerance, and human trials are warranted.

The computational model now has been implemented in JAVA, and is available for use by other investigators via the Web (http://knut.kumoh.ac.kr/~mech/cvsim/Simulator.html).

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Michael D. Schneider, M.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Cardiac Atrophy**
FUNDING:	**$140,000 (FY 1998); $143,051 (FY 1999); $143,050 (FY 2000)**
TOTAL FUNDING:	**$426,101**

PROJECT EXECUTIVE SUMMARY

Key findings and discoveries:

1. The requisite physiological and molecular methods were implemented to assess cardiac function in mouse and rat hearts in response to cardiac loading and unloading, including M-mode and Doppler echocardiographic assessment of cardiac structure and function in mice, and in vivo left ventricular hemodynamic recordings.
2. Programmed cell death (apoptosis) in cardiac myocytes was demonstrated in response to altered load. Multiple complementary approaches to monitor and alleviate apoptosis were investigated.
3. Contractility is impaired in mouse myocytes with down-regulation of SERCA2, a gene whose expression is defective, following decreased loading.
4. Heterotopic transplantation, the best-established model of cardiac unloading, was implented successfully. Unloading by this means was shown to induce cardiac atrophy, down-regulation of SERCA2, and up-regulation of nominal markers of hypertrophy (despite the opposite effect on growth).
5. Contractile reserve was depressed in myocytes from unloaded hearts. Impaired contractile reserve in myocytes from unloaded hearts is related to the inability to augment intracellular systolic Ca_{2+} during this challenge.
6. Growth hormone is a potential countermeasure, which partials rescues SERCA2 expression during cardiac atrophy, but higher doses than tested will be required to correct the defects in contractile reserve and cardiac mass.
7. Biochemical studies to identify novel load-regulated proteins, for mechanistic insights and as potential sites for intervention, led to two fundamental discoveries. Load regulates activation of RNA polymerase II, which controls global rates of RNA synthesis per cell, via the protein kinase, Cdk7. Load also regulates the mitogen-activated protein kinase, TAK1, which mediates (in part) the effects of load on cell survival and gene expression.
8. Genetic studies to identify load-regulated genes, for mechanistic insights and as potential sites for intervention, led to the discovery of more than 50 differentially expressed genes, beyond those that have been reported previously to be targets of load, including many signaling proteins: Rap1B, protein phosphatase 1γ [PP1γ], inhibitor protein phosphatase 2A [IPP2A], mss4, dynamin-like protein 1 [DLP-1], and the putative mechanosensor, ILK.

Satisfaction of the hypotheses, technology, objectives and aims:

The cardiovascular system undergoes multiple changes during prolonged space-flight as adaptation to the microgravity environment. During spaceflight, the cardiovascular system is not subjected to the biomechanical stresses associated with changes in posture in a gravitational field. Space-flight is associated with a modest reduction in intravascular volume and red blood cell mass, a decrease in arterial blood pressure, and a relative shift of intravascular volume from the lower body to the thorax and head; importantly, these adaptations occur even in the presence

of regular exercise regimens and hydration during space-flight missions. It is recognized that the integrated cardiovascular adaptation to prolonged spaceflight may be in the net beneficial in the microgravity environment, but may be maladaptive when the cardiovascular system is subjected to severe abrupt stresses such as reentry into a higher gravitational field or the requirement to perform sustained and near-maximal exercise. In the present grant period, the NSBRI Cardiovascular Alterations team addressed three potential critical risks that may be imposed during long duration spaceflight. Each of these potential critical risks requires the elucidation of mechanisms in order to develop rational and effective countermeasures that can be applied in human long-duration spaceflight. The critical risks include: (1) the development of orthostatic hypotension and risk of syncope upon reentry into the earth (and potentially Mars) gravitational field; (2) the susceptibility to rhythm disturbances; and (3) the reduction in cardiac mass.

Our team focused specifically on the problem of changes in cardiac remodeling and gene expression which occur in response to cardiac unloading (cardiac atrophy) in rodent models, and compared these observations with changes that occur in response to excess load (cardiac hypertrophy). The observations in this grant period support our index hypothesis that the plasticity of the heart to adapt to perturbations in load is limited, and that cardiac unloading stimulates changes in gene expression which are phenotypic of cardiac hypertrophy. In short, directionally similar changes in gene expression occur during BOTH increases and decreases in cardiac muscle cell size, indicating these are reflective of cell remodeling per se.

This paradigm shift has important predictive implications for future hypothesis-testing and for specific counter-measures, as a number of adverse pathways affecting contractility, cardiac compliance, and even cell survival are activated as part of the known hypertrophic gene program.

The significance of this work, directed at cardiac atrophy, was highlighted by preliminary human data, made known during the study period by Dr. J. Yelle, Head of the Cardiovascular Laboratory of the Johnson Space Center. Whereas no reduction in LV mass was found using echocardiography, after short-duration NASA missions (n=13), a significant, reproducible, 10% loss of LV mass was seen in long-duration MIR missions (n=4). This compels greater diligence, in monitoring the long-term effects of microgravity on cardiac mass in astronauts, and also reinforces the scientific need to understand the mechanisms and molecular details, underlying this gross change.

Thus, the overall hypotheses posed by the investigators have gained substantial reinforcement from both animal and human studies.

Implications for risk reduction related to the Critical Research Path and Earth medical problems:

The whole-animal and isolated-cell studies together demonstrate, unambiguously, loss of cardiac mass, alterations of normal gene expression, and impaired contractile reserve. Thus, further studies of cardiac atrophy during unloading are clearly warranted.

Growth hormone can partially rescue impaired expression of SERCA2, encoding the calcium "pump," in this model of cardiac atrophy. Thus, further studies of growth hormone are clearly warranted, at higher dosages and in concert with other countermeasures.

Mechanical load affects numerous targets that had never previously been identified but were disclosed by novel biochemical and genetic methods. Thus, future work is needed to explore the functional contribution of these candidate effectors, to develop countermeasures for those, like

TAK1, whose consequences are adverse, and, conversely, to develop therapies based on those, like Cdk7, whose effects on cardiac mass or function is shown to be beneficial.

RESEARCH AREA:	**Cardiovascular Alterations**
PRINCIPALINVESTIGATOR:	**Richard J. Cohen, M.D., Ph.D.**
ORGANIZATION:	**Massachusetts Institute of Technology**
PROJECT TITLE:	**Non-Invasive Assessment of Susceptibility To Ventricular Arrhythmias During Simulated Microgravity**
FUNDING:	**$10,800 (FY 1998); $22,118 (FY 1999); $22,118 (FY 2000)**
TOTAL FUNDING:	**$55,036**

PROJECT EXECUTIVE SUMMARY

The Cardiovascular Alterations Team has conducted studies to determine what alterations in hemodynamic regulation result from sixteen days of simulated microgravity exposure in normal human subjects. In this project we made additional measurements on these same study subjects in order to determine whether there is an increase in susceptibility to ventricular arrhythmias resulting from simulated microgravity exposure.

Numerous anecdotal and documented reports from the past 30 years suggest that the incidence of ventricular arrhythmias among astronauts is increased during space flight. For example, documented runs of ventricular tachycardia have been recorded from crew members of Skylab and Mir, there was much attention given by the lay press to Mir Commander Vasily Tsibliyev's complaints of heart rhythm irregularities in July of 1997, and cardiovascular mechanisms may have been causal in the recent death of an experimental primate shortly after return from space. In 1986, a Mir cosmonaut, Alexander Laveikin, was brought home and replaced with an alternate cosmonaut as a result of cardiac dysrhythmias that began during extravehicular activity. Furthermore, at a joint NASA/NSBRI workshop held in January 1998, cardiac arrhythmias were identified as the highest priority cardiovascular risk to a human Mars mission. Despite the evidence for the risk of a potentially lethal arrhythmia resulting from microgravity exposure, the effects of space flight and the associated physiologic stresses on cardiac conduction processes are not known, and an increase in cardiac susceptibility to arrhythmias has never been quantified.

In this study we found that 16 days of head down bed rest appears to increase the incidence of microvolt level T wave alternans, which reverts to baseline levels 2-3 days after the bed rest period. This is the first data obtained under control conditions which indicates that simulated microgravity alters cardiac electrical processes. The presence of T wave alternans (although with a lower onset heart rate than observed) in clinical patient populations has been found to indicate an increased risk of ventricular arrhythmias.

The data presented here indicate the need to further investigate the effect of space flight on the heart's susceptiblity to ventricular arrhythmias, and if necessary develop appropriate countermeasures.

Microvolt level T wave alternans testing developed under NASA and NSBRI support has now been successfully commercialized and was FDA cleared in April 1999 as a non-invasive means of identifying patients at increased risk of ventricular arrhythmias and sudden cardiac death. Three hundred thousand Americans die each year of sudden cardiac death. Effective treatment is available in the form of the implantable cardioverter/defibrillator. The problem has been that until now there has not been an effective means of identifying who is at risk. T wave alternans testing is now in clinical use and promises to have a major role in reducing sudden cardiac death.

NSBRI RESEARCH PROGRAM
HUMAN PERFORMANCE FACTORS

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HUMAN PERFORMANCE FACTORS TEAM PROGRAM EXECUTIVE SUMMARY

Errors in human performance cause most accidents in technology-rich environments such as commercial aviation, where two-thirds of accidents are attributable to performance errors by cockpit crews. In space, the contribution of human performance factors to mission success is even greater, since a number of fundamental aspects of the space environment compromise physiologic systems critically involved in human performance. Human factors is a broad area that includes biological limits on performance [e.g., circadian rhythms, sleep need, microgravity, radiation, environmental factors (temperature)], operational demands on performance (e.g., skilled task demands, monitoring complex automation, mission requirements), psychosocial effects on performance (e.g., effects of isolation, crew selection, family contact, crew communication/coordination) and human ergonomics (e.g., habitability, equipment design, workload, training). Initially, the Human Performance Factors, Sleep and Chronobiology Team is focused on the biological limits of human performance, particularly those compromised by specific aspects of the space environment, including microgravity, an absence of geophysical 24-h cycles, limited sleep/rest opportunities, a high level of automation and a remote, inaccessible location. Such conditions will likely be ubiquitous among the astronauts and are known to affect physiologic, behavioral and cognitive processes critically involved in human performance. These aspects of the space environment result in or require: (a) disrupted circadian entrainment; (b) dyssomnia; (c) cumulative sleep loss; (d) execution of life science research remote from the Principal Investigator (PI).

The overall strategy of the Human Performance Factors, Sleep and Chronobiology Team was based on the recognition that optimizing human performance in space can best be achieved by: (1) understanding the basic mechanisms underlying the deterioration of human neurobehavioral function in space related to these factors; and (2) developing effective countermeasures based on those mechanisms to minimize human error and optimize human performance in the highly automated space environment. Currently, for example, astronauts' sleep duration, which is one of the most fundamental determinants of their waking neurobehavioral performance, averages only 6 hours per night, and may be as low as 3 to 4 hours per night. Ground-based studies indicate that within 2 weeks, the effects of such cumulative sleep deprivation are equivalent to the effects of 48-60 hours of total sleep deprivation. Recent work elsewhere indicates that as little as 24 hours of total sleep deprivation has been reported to degrade aspects of neurobehavioral performance to a level comparable to a blood alcohol level of 0.10 percent. To counteract their difficulty sleeping, astronauts and the flight surgeons responsible for their medical care currently rely during space flight on *ad lib* self-administration of hypnotic medications that were developed for the treatment of insomnia, with 50% of crew members in dual shift operations resorting to sleeping pill use during the missions.

This integrated research team has investigated a series of novel approaches to address such human performance factors, including: the mechanisms of circadian entrainment and sleep regulation; statistical algorithms for on-line analysis of physiologic variables monitored during long-duration space missions; and the development of expert systems for the remote execution of life science experiments. These approaches were integrated with the aim of developing countermeasures and testing their efficacy. The multi-disciplinary approach adopted for study of the affected physiologic, behavioral and cognitive processes in humans (i.e., including circadian entrainment, sleep homeostasis, and decision-making processes) incorporated five team projects and two inter-team synergy projects.

Integration was achieved by thematic organization around the defining characteristics of the space environment that influence human performance. The research program of the Human Performance Factors, Sleep and Chronobiology Team was a goal-directed research program that provided an integrated contribution to the overall NSBRI mission and addressed the Institute's Aims and Objectives by: (1) Designing, implementing, and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight; (2) Defining the whole-organism integrated-physiological and neurobehavioral responses that ultimately determine these impediments and designing novel countermeasures based on these responses; (3) Establishing support technologies to maximize human performance in space and to reduce the probability of human performance failure; (4) Transferring and disseminating the advances in knowledge and technology acquired to populations on earth in which performance is jeopardized; (5) Providing training of new scientists in the space life sciences by recruiting young scientists for active participation in the proposed ground-based research program and by mentoring these young scientists for the duration of these projects.

The research that the team has conducted is relevant for the round-the-clock work schedules (day, evening and night work) on the International Space Station, the altered sleep/wake schedule required on a Mars surface station, or any other situation in which the work-rest schedule is shifted or sleep loss is incurred. It also has relevance for ground personnel monitoring orbiting crew members who must do so around-the-clock. Through the efforts of this Program, the Human Performance, Sleep and Chronobiology Team has worked towards developing effective countermeasures to minimize human error and optimize human performance in the highly automated space environment. The research program of the Human Performance Factors, Sleep and Chronobiology Team has been a goal-directed research program that has provided an integrated contribution to the overall NSBRI mission. The results of this team effort could have an important effect on the health, safety and productivity of astronauts during extended duration missions, such as those planned for the International Space Station and for the manned mission to Mars.

RESEARCH AREA:	**Human Performance Factors**
PRINCIPAL INVESTIGATOR:	**Charles A. Czeisler, M.D., Ph.D.**
ORGANIZATION:	**Harvard Medical School and Brigham and Women's Hospital**
PROJECT TITLE:	**Circadian Entrainment, Sleep-Wake Regulation and Neurobehavioral Performance During Extended Duration Space Flight**
FUNDING:	**$390,577 (FY 1998); $416,885 (FY 1999), $416,885 (FY 2000)**
TOTAL FUNDING:	**$1,224,347**

PROJECT EXECUTIVE SUMMARY

Long-duration exploration class space flight requires crew members to maintain a high level of cognitive performance and vigilance while operating and monitoring sophisticated instrumentation. However, the reduction in the strength of environmental synchronizers in the space environment leads to misalignment of circadian phase among crew members, coupled with restricted time available to sleep, results in sleep deprivation and consequent deterioration of neurobehavioral function.

Crew members are provided, and presently use, long-acting benzodiazepine hypnotics on board the current, relatively brief space shuttle missions to counteract such sleep disruption, a situation that is only likely to worsen during extended duration missions. Given the known carry-over effects of such compounds on daytime performance, together with the reduction in emergency readiness associated with their use at night, NASA has recognized the need to develop effective but safe countermeasures to allow crew members to obtain an adequate amount of sleep. Over the past nine years, we have successfully implemented a new technology for shuttle crew members involving bright light exposure during the pre-launch period to facilitate adaptation of the circadian timing system to the inversions of the sleep-wake schedule often required during dual shift missions. However for long duration space station missions it will be necessary to develop effective and attainable countermeasures that can be used chronically to optimize circadian entrainment.

Our current research effort is to study the effects of light-dark cycles with reduced *zeitgeber* strength, such as are anticipated during exploration class space missions, on the entrainment of the endogenous circadian timing system and to study the effects of a countermeasure that consists of scheduled brief exposures to bright light on the human circadian timing system. The studies are designed to address the following Specific Aims:

1) test the hypothesis that synchronization of the human circadian pacemaker will be disturbed in men and women by the reduction in LD cycle strength.

2) test the hypothesis that this disturbed circadian synchronization will result in the secretion of the sleep-promoting hormone melatonin during the waking day, disturbed sleep, reduced growth hormone secretion, and impaired performance and daytime alertness;

Results suggest that a strictly scheduled wake-sleep cycle with dim light levels, similar to that which astronauts are currently exposed on space shuttle missions, is sufficient to maintain entrainment of the human circadian pacemaker to the 24.0 hr day for most but not all subjects tested. No subjects entrained to a longer-than-24-hr day under similar conditions. Circadian

misalignment resulted in disturbed sleep, impaired alertness and performance, secretion of melatonin during the waking day and reduced nocturnal growth hormone secretion. Preliminary studies suggest that stronger synchronizers, such as brighter light, will be necessary to entrain the longer-than-24-hour intrinsic circadian period of all humans to the 24.0 day and other day lengths such as the ~24.65 solar day of Mars.

The results of the current research may have important implications for the treatment of circadian rhythm sleep disorders, such as delayed sleep phase syndrome and shift-work dyssomnia, which are anticipated to have a high incidence and prevalence during extended duration space flight such as planned for the International Space Station and astronaut missions to Mars.

RESEARCH AREA:	**Human Performance Factors**
PRINCIPAL INVESTIGATOR:	**David F. Dinges, Ph.D.**
ORGANIZATION:	**University of Pennsylvania**
PROJECT TITLE:	**Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight**
FUNDING:	**$297,700 (FY 1998); $313,992 (FY 1999); $314,306 (FY 2000)**
TOTAL FUNDING:	**$925,998**

PROJECT EXECUTIVE SUMMARY

This project is concerned with identifying ways to prevent neurobehavioral and physical deterioration due to inadequate sleep in astronauts during long-duration manned space flight. The performance capability of astronauts during extended-duration space flight depends heavily on achieving recovery through adequate sleep. Even with appropriate circadian alignment, sleep loss can erode fundamental elements of human performance capability including vigilance, cognitive speed and accuracy, working memory, reaction time, and physiological alertness. Adequate sleep is essential during manned space flight not only to ensure high levels of safe and effective human performance, but also as a basic regulatory biology critical to healthy human functioning.

There is now extensive objective evidence that astronaut sleep is frequently restricted in space flight to averages between 4 hr and 6.5 hr/day. Chronic sleep restriction during manned space flight can occur in response to endogenous disturbances of sleep (microgravity, motion sickness, stress, circadian rhythms), environmental disruptions of sleep (noise, temperature, light), and curtailment of sleep due to the work demands and other activities that accompany extended space flight operations. The mechanism through which this risk emerges is the development of cumulative homeostatic pressure for sleep across consecutive days of inadequate sleep. Research has shown that the physiological sleepiness and performance deficits engendered by sleep debt can progressively worsen (i.e., accumulate) over consecutive days of sleep restriction, and that sleep limited to levels commonly experienced by astronauts (i.e., 4 - 6hr per night) for as little as 1 week, can result in increased lapses of attention, degradation of response times, deficits in complex problem solving, reduced learning, mood disturbance, and disruption of essential neuroendocrine functions.

The prevention of cumulative performance deficits and neuroendocrine disruption from sleep restriction during extended duration space flight involves finding the most effective ways to obtain sleep in order to maintain the high-level cognitive and physical performance functions required for manned space flight. There is currently a critical deficiency in knowledge of the effects of how variations in sleep duration and timing relate to the most efficient return of performance per unit time invested in sleep during long-duration missions, and how the nature of sleep physiology changes as a function of sleep restriction and performance degradation. The primary aim of this project is to meet these critical deficiencies through utilization of a response surface experimental paradigm, testing in a dose-response manner, varying combinations of sleep duration and timing, for the purpose of establishing how to most effectively limit the cumulative adverse effects on human performance and physiology of chronic sleep restriction in space operations.

To develop a response surface models of the best sleep-wake schedules for astronauts, 90 healthy men and women underwent a 14-day ground-based laboratory protocol involving random assignment to one of 18 sleep-ration cells, each involving the same sleep ration for 10 consecutive days. The sleep-ration assignments involved four nocturnal anchor sleep durations (4.2, 5.2, 6.2, 8.2 hr) and six diurnal nap sleep durations (0.4, 0.8, 1.2, 1.6, 2.0, 2.4 hr) crossed to yield a total of four anchor-sleep-only conditions, and 14 anchor + nap sleep conditions, and spanning a dynamic range of cumulative sleep debts (i.e., from 0 to 40 hr in a 10-day period). Throughout the 14 days, subjects lived in conditions that simulated aspects of prolonged space flight (e.g., confined small groups, social and environmental isolation, controlled diet and activity) and underwent a wide range of quasi-continuous neurobehavioral performance tests and continuous physiological monitoring of brain activity, sleep physiology, core body temperature, and behavioral motility. The laboratory environment was designed to simulate the low light, tight quarters, and lack of social contact with the outside world that will characterize long-duration space flight.

Data acquisition in this project has been completed response surface model (RSM) development and hypothesis testing on the large set of neurobehavioral and physiological outcomes are currently underway. Thus far, the results of the experiment have revealed that subjects are able to achieve significant levels of physiological sleep when both anchor (nocturnal) and nap (diurnal) sleep opportunities are chronic (i.e., part of a daily schedule), regardless of the time in bed allowed for sleep. Even daytime nap opportunities as brief as 0.4 hr (24 minutes) consistently resulted in physiological sleep. This indicates that the diverse range of restricted anchor + nap sleep durations tested in this protocol will likely result in sleep if used by astronauts. Response surface models fit to neurobehavioral data reveal that the combination of a restricted duration anchor sleep and a diurnal nap can help prevent the development of cumulative deficits that can occur when only restricted anchor sleep is permitted (as is currently typical in space flight). Moreover, all response surface models being evaluated for optimizing performance, mood, sleep physiology, and hormonal profiles in the face of restricted total sleep time include the duration of nocturnal anchor sleep, the duration of diurnal nap sleep, age, gender, and baseline individual differences. Thus, the results of this study will permit us to estimate the relative contributions of astronaut demographics (age, gender) and individual abilities (baseline differences) to cumulative neurobehavioral deficits due to sleep restriction. This permits a more precise estimate of how a given sleep-wake schedule will likely affect different crews.

This experiment is the first ground-based study to utilize the slopes of cumulative neurobehavioral deficits and physiological changes across days of chronic sleep restriction, to determine the extent to which the duration of sleep per 24 hours (in the range commonly experienced by astronauts in flight) and the use of combined anchor + nap sleep opportunities each day, can prevent or attenuate the development of cumulative fatigue and performance deficits. The response surface experimental paradigm affords a high return of information regarding the optimal way to utilize sleep in operations that inherently limit time for sleep in the space flight environment. The results of the proposed research will contribute to the optimization of performance, productivity, safety and health during extended missions, by providing astronauts with the most efficient sleep-wake schedules. The results of this project also have important implications for optimizing work-rest scheduling in Earth-based safety-sensitive industries that must operate around the clock (e.g., transportation, military, public safety).

Finally, the research project will also help address critical questions pertaining to human performance failure in space due to sleep and circadian problems. Thus, the results of this project will help determine both the acute and long-term neurobehavioral and physiological effects of exposure to restricted sleep durations in the range commonly experienced by astronauts in space flight. It will establish whether sleep-wake schedule countermeasures involving varying combinations of restricted anchor sleep and nap sleep can effectively mitigate the performance risks posed to astronauts by chronically restricted sleep in space flight. The project will also provide estimates of the long-term effects of optimal sleep schedule countermeasures on hormonal profiles, sleep inertia and related physiological and neurobehavioral functions. Finally, the project is providing performance technologies and needed data for the development of a biomathematical model of human performance capability relative to sleep schedules and circadian physiology. These techniques will ultimately aid astronauts in the self-management of sleep and alertness during long-duration space flight.

RESEARCH AREA:	**Human Performance Factors**
PRINCIPAL INVESTIGATOR:	**Derk-Jan Dijk, Ph.D.**
ORGANIZATION:	**Harvard Medical School and Brigham and Women's Hospital**
PROJECT TITLE:	**Quantitative EEG Monitoring of Vigilance: Effects of Sleep Deprivation, Circadian Phase and Sympathetic Activation**
FUNDING:	**$137,652 (FY 1998); $121,497 (FY 1999); $121,497 (FY 2000)**
TOTAL FUNDING:	**$380,646**

PROJECT EXECUTIVE SUMMARY

Shuttle astronauts typically sleep only 6 to 6.5 hours per day while in orbit. This sleep loss is related to recurrent sleep cycle shifting – due to mission-dependent orbital mechanics and mission duration requirements – and associated circadian displacement of sleep, the operational demands of space flight, noise and space motion sickness. Such sleep schedules are known to produce poor subjective sleep quality, daytime sleepiness, reduced attention, negative mood, slower reaction times, and impaired daytime alertness. Countermeasures to allow crew members to obtain an adequate amount of sleep and maintain adequate levels of neurobehavioral performance are being developed and investigated. However, it is necessary to develop methods that allow effective and attainable in-flight monitoring of vigilance to evaluate the effectiveness of these countermeasures and to detect and predict online critical decrements in alertness/performance. There is growing evidence to indicate that sleep loss and associated decrements in neurobehavioral function are reflected in the spectral composition of the electroencephalogram (EEG) during wakefulness as well as in the incidence of slow eye movements recorded by the electro-oculogram (EOG). Furthermore, our preliminary data indicated that these changes in the EEG during wakefulness are more pronounced when subjects are in a supine posture, which mimicks some of the physiologic effects of microgravity. Therefore, we evaluated the following hypotheses: (1) that during a 40-h period of wakefulness (i.e., one night of total sleep deprivation) neurobehavioral function deteriorates, the incidence of slow eye-movements and EEG power density in the theta frequencies increases especially in frontal areas of the brain; (2) that the sleep deprivation induced deterioration of neurobehavioral function and changes in the incidence of slow eye movements and the spectral composition of the EEG are more pronounced when subjects are in a supine position; and (3) that based on assessment of slow-eye movements and quantitative on-line topographical analyses of EEG during wakefulness an EEG and or EOG parameter can be derived/constructed which accurately predicts changes in neurobehavioral function.

In a series of experiments and data analysis projects conducted during the first three years of this project we have established that:

1. The spectral composition of the EEG during wakefulness exhibits pronounced and predictable changes during a 24-h period of sustained wakefulness.
2. The changes associated with sleep loss are most pronounced in EEGs derived from frontal areas of the brain, and in particular so in the delta and theta frequencies, both during wakefulness and during sleep.
3. Changes in alertness and psychomotor vigilance correlate with changes in EEG power density in the delta and theta frequencies in frontal derivations.
4. The incidence of slow eye movements during wakefulness increases during sleep loss and correlates with changes in alertness and psychomotor vigilance. This correlation is so

tight that inter-individual differences in the time course of the incidence of slow eye movements closely resemble the inter-individual differences in the time course of neurobehavioral performance during a 24-h episode of sustained wakefulness.

5. The circadian pacemaker modulates the incidence of slow eye movements as well as the spectral composition of the EEG during wakefulness.
6. Light-induced changes in the amplitude of the circadian pacemaker and associated changes in the amplitude of the circadian modulation of alertness are associated with changes in the amplitude of the circadian modulation of the incidence of slow eye movements
7. Light-induced acute changes in alertness are associated with acute changes in the EEG as well as with the incidence of slow eye movements during wakefulness.
8. Posture modulates the apparent amplitude of the circadian rhythm of body temperature and heart rate such that this amplitude is reduced when subjects are in a supine posture during 40-h of wakefulness.
9. Posture modulates the effects of sleep loss and circadian phase on neurobehavioral performance as assessed by the psychomotor vigilance test such that the detrimental effects of sleep loss/circadian phase are more pronounced when subjects are in a supine posture during 40-h of wakefulness.
10. The incidence of slow-eye movements during a drowsiness test predicts performance on a psychomotor vigilance task conducted one hour later.

These data, generated by the hypotheses described above as well as by secondary hypotheses, establish that the original hypotheses, specific aims and their modifications as described in the original proposal and subsequent progress reports, were fruitful. These new findings establish a close and robust association of frontal EEG and ocular parameters with changes in neurobehavioral performance in a variety of protocols in which sleep homeostasis and circadian rhythmicity were manipulated. Circadian rhythmicity and sleep homeostasis have been established to be major determinants of performance and our data establish that they are also major determinants of the waking EEG and ocular parameters. This implies that these parameters are likely to be associated with performance in a variety of conditions in which performance is jeopardized by changes in the status of the sleep homeostat or changes in circadian phase.

Furthermore, these data indicate that EEG/EOG based on-line monitoring of alertness/performance can serve as a practical and attainable tool to predict and prevent critical decrements in performance and alertness, without the need to conduct time consuming tests of neurobehavioral performance.

The research conducted in the current grant period aimed at the development of countermeasures for Human performance failure because of sleep and circadian rhythm problems [Risk 19, Critical Road Map http://criticalpath.jsc.nasa.gov]. In particular, our research relates to the critical questions 6.08 (What are the best methods for monitoring the status of sleep and circadian functioning and for assessing the effects of sleep loss and circadian dysrhythmia that are also portable and non-intrusive in the spaceflight environment?) and 6.21 (What mathematical and experimental models best predict performance problems associated with sleep-wake and work history and circadian rhythm status, and also provide guidelines for successful countermeasure strategies?). In addition, our research is relevant to critical question 6.05 (What are the acute and long-term effects of exposure to the space environment on biological rhythmicity, on sleep architecture, quality, and quantity, and their relationship to performance capability?) and 6.06 (Which countermeasure or combination of behavioral and physiological

countermeasures will optimally mitigate specific performance problems associated with sleep loss and circadian disturbances during a Mars mission?).

Further understanding of the relationship between EEG/EOG and neurobehavioral function could thus have a profound effect on the health, productivity and safety of astronauts during space missions.

The research is relevant for the round-the-clock work schedules (day, evening and night work) on the International Space Station, the altered sleep/wake schedule on a Mars surface station, or any other situation where the work-rest schedule is shifted and sleep loss is incurred. It also has relevance for ground personnel monitoring orbiting crew members who must do so working round-the-clock schedules.

RESEARCH AREA:	**Human Performance Factors**
PRINCIPAL INVESTIGATOR:	**Emery N. Brown, M.D., Ph.D.**
ORGANIZATION:	**Harvard Medical School and Massachusetts General Hospital**
PROJECT TITLE:	**On-Line Analysis of Physiologic and Neurobehavioral Variables During Long-Duration Space Missions**
FUNDING:	**$104,027 (FY 1998); $99,953 (FY 1999); $99,953 (FY 2000)**
TOTAL FUNDING:	**$303,933**

PROJECT EXECUTIVE SUMMARY

BACKGROUND

The goal of this project is to develop reliable statistical algorithms for on-line analysis of physiologic and neurobehavioral variables monitored during long-duration space missions. Maintenance of physiologic and neurobehavioral homeostasis during long-duration space missions is crucial for ensuring optimal crew performance. If countermeasures are not applied, alterations in homeostasis will occur in nearly all-physiologic systems. During such missions data from most of these systems will be either continually and/or continuously monitored. Therefore, if these data can be analyzed as they are acquired and the status of these systems can be continually assessed, then once alterations are detected, appropriate countermeasures can be applied to correct them.

One of the most important physiologic systems in which to maintain homeostasis during long-duration missions is the circadian system. To detect and treat alterations in circadian physiology during long duration space missions requires development of: 1) a ground-based protocol to assess the status of the circadian system under the light-dark environment in which crews in space will typically work; and 2) appropriate statistical methods to make this assessment. The protocol in Project 1, Circadian Entrainment, Sleep-Wake Regulation and Neurobehavioral will study human volunteers under the simulated light-dark environment of long-duration space missions. Therefore, we propose to develop statistical models to characterize in near real time circadian and neurobehavioral physiology under these conditions.

The specific aims of this project are to test the hypotheses that: 1) Dynamic statistical methods based on the Kronauer model of the human circadian system can be developed to estimate circadian phase, period, amplitude from core-temperature data collected under simulated light-dark conditions of long-duration space missions. 2) Analytic formulae and numerical algorithms can be developed to compute the error in the estimates of circadian phase, period and amplitude determined from the data in Specific Aim 1. 3) Statistical models can detect reliably in near real-time (daily) significant alternations in the circadian physiology of individual subjects by analyzing the circadian and neurobehavioral data collected in Project 1. 4) Criteria can be developed using the Kronauer model and the recently developed Jewett model of cognitive performance and subjective alertness to define altered circadian and neurobehavioral physiology and to set conditions for immediate administration of countermeasures.

RESEARCH PLAN YEARS 2 AND 3

At the outset of Year 2 we made three changes in the research plan as a consequence of the research findings in Year 1 and the recommendations of the review committee.

Change 1: Dynamic Assessments of Circadian Phase from Forced Desynchrony Studies. In our Year 1 research plan, our original goal was to use the data collected during the 25 24 hour days

of core-temperature data collected from Project 1 to develop a technique for making dynamic assessments of circadian phase. These estimates would provide the circadian input to the performance and subjective alertness model prediction developed by Dr. Jewett. Our original hypothesis was that under low light conditions these subjects would free run and therefore these data would provide an excellent framework for making dynamic assessments of circadian phase. All of the 3 subjects analyzed by the end of Year 1 were entrained during the 25 24 hour day. Our analysis and the independent constant routine assessments confirmed this. Therefore to test the ability of our analytic framework to make dynamic assessments of circadian phase we use the temperature data from the forced desynchrony part of the protocol. During this phase of the protocol the subject is desynchronized from the 28-hour day.

Change 2: Average Prediction of Performance and Subjective Alertness. In our Year 1 research plan our original goal was to develop straight away an algorithm for making time specific individual predictions of performance and subjective alertness using the models developed by Dr. Jewett. We realized that moving directly to individual predictions was too large an initial step. Therefore we will use the performance, alertness and circadian phase data to first adapt the Jewett model to predict average performance, since this is what it was initially developed to predict. Once the model shows good predictions with average performance and subjective alertness, we will then return the problem of individual predictions.

Change 3: Using the Expertise of a Neurobehavioralist on the Project 4. Our scientific review committee recommended that we include a neurobehavioralist on our team in order to better focus the work on performance and subjective alertness. In response to this suggestion, we have Dr. Megan Jewett working on this component of the modeling for the project. She developed the performance and subjective alertness models for her Ph.D. dissertation and has been working with us to adapt them to the study of the subjects on the simulated long-duration space missions.

The objective in Year 3 was to analyze the core-temperature, performance and alertness data of the 7 subjects in the control group from Project 1.

PROGRESS, RESULTS AND IMPLICATIONS FOR FUTURE RESEARCH

Core-Temperature Analysis. The methods were applied to the 7 control subjects studied in Project 1. We have successfully used our methods to analyze core-temperature data on the forced desynchrony protocol and demonstrate that the period of the human circadian pacemaker is closer to 24 instead of 25 hours. We published these findings in *Science* in July of 1999. In addition we published two manuscripts detailing our methods for dynamic assessment of circadian phase.

Genetic Algorithm. We made the genetic algorithm a standard part of our analysis framework. It has been implemented with a continuous state discrete-time Kalman filter algorithm in order to fit unevenly spaced core-temperature data.

New Model for Core-Temperature. The new core-temperature model described in our two publications holds promise for giving a better description of the dynamics of the human circadian pacemaker. This description can be further enhanced if realistic models of the thermoregulatory and activity interactions with the circadian pacemaker can be characterized. We will work on developing these model components.

Analysis of Performance and Subjective Alertness. We are completing our analysis of subjective alertness and performance for all of the subjects in the control group in Project 1. A manuscript on this work in under preparation.

Growth Hormone Model. We have developed a growth hormone model so that we can now include the negative feedback to measure the effect of growth hormone plasma levels on its own secretion. We are using the model to analyze normal growth hormone physiology in normal subjects.

Cortisol Model. We have also submitted for publication a manuscript detailing a new stochastic differential equation model of plasma cortisol levels. This model may be used to analyze diurnal cortisol patterns as well as serving as a starting point for extending our work on growth hormone to the analysis of melatonin series with more than one secretory event.

Implications for Future Research. We have developed more accurate statistical models of human circadian marker rhythms including core-temperature, growth hormone and cortisol. These new models provide an accurate means of assessing characterizing circadian physiology with respect to standard marker rhythms in both space and non-space related research. We have used the dynamic phase information from the core-temperature model as an input to the Jewett performance and subjective alertness models. The paradigm may be useful for developing accurate strategies to monitor the circadian health, neurobehavioral state of astronauts during long-duration space missions and for implementing and measuring the effects of countermeasures when significant alterations in these states are detected.

RESEARCH AREA:	**Human Performance Factors**
PRINCIPAL INVESTIGATOR:	**Laurence R. Young, Sc.D.**
ORGANIZATION:	**Massachusetts Institute of Technology**
PROJECT TITLE:	**Principal Investigator-in-a-Box**
FUNDING:	**$30,000 (FY 1998); $61,433 (FY 1999); $61,204 (FY 2000)**
TOTAL FUNDING:	**$152,637**

PROJECT EXECUTIVE SUMMARY

The efficiency of crew performance on the ISS will depend critically on training and coaching, both before launch and during a flight increment. This project investigated the ability of a real-time expert system to improve performance and reduce the time needed to diagnose errors and troubleshoot a space life science experiment. The experiment tested the efficacy of *Principal Investigator-in-a-Box*, or [PI], a tool for assisting relatively untrained "astronaut surrogates" to detect, diagnose, and correct realistic instrumentation anomalies. It is the first evaluation of such a decision aid under controlled conditions, and will help determine the applicability of expert systems in future space flight research. Two groups of subjects, receiving identical training on sleep monitoring, were tested on two days. Half the subjects were tested with [PI] assistance only on Day 1; the other half only on Day 2. For all subjects, time to detect and identify randomly introduced artifacts and to complete a normal sleep monitoring calibration was measured with and without [PI]. The expert system rules build on the existing [PI] software for troubleshooting and error detection developed for the Neurolab sleep experiment.

Key Findings

Results from the study indicate that an expert system can be used for fault management in a space life science experiment. Furthermore, astronauts who used [PI] during missions found it to be a useful decision aid. However, its utility depends, at least in part, on training and the user's computer literacy.

The feasibility of the PI-in-a-Box concept has been shown in ground studies as well, confirming the favorable experience with it in space. With appropriate training, [PI] reduced time to detect and time to correctly troubleshoot faults in a sleep instrumentation setup. We found that by observing the reliability of the indicator lights, [PI] was helpful for subjects on Day 1, and was a hindrance for them on Day 2. There were also fewer undetected anomalies and undiagnosed faults with [PI] than without it.

Satisfaction of Hypotheses

As stated in the proposal, our hypothesis is that use of a computer decision aid (PI-in-a-Box) will improve experiment performance on three independent measures, compared to the same subjects' performance without the decision aid. These independent measures are:

1. Average time to detect the deterioration of signal quality to beyond a pre-determined level,
2. Average time to identify correctly the source of unanalyzable data in a complex situation with several alternative causes,
3. Average time to complete a normal calibration and run a physiological experiment.

The pilot study and observations of Neurolab and STS-95 data were used to evaluate [PI]'s ability to help with detection times for anomalous signals. The results of the pilot study showed that [PI] assistance reduced the detection time, though not by a statistically significant amount. Training, or the cross effect of [PI] and Day, was found to be significant. The study also found that the number of undetected anomalies was significantly lower when [PI] was available. Gender effects were also found to be significant for the detection task.

The Neurolab and STS-95 data were comprised of signal recordings of the first few minutes of each instrumentation session. It was found that [PI] correctly detected 84% of the anomalies that were not saturated in the signals from the Neurolab data. In the STS-95 data, [PI] correctly detected 86% of all signal anomalies. Overall, the cardiorespiratory indicator lights were the most reliable, while the electroencephalogram (EEG), and electro-oculogram (EOG) signals were the most prone to false alarms from [PI] indicator lights.

The study completed in Phase I showed that the use of [PI] assistance has a different impact for different types of stimulus files. It seems to neutralize differences between signal anomalies of different simulation files. Furthermore, in Phase 2, subjects allowed fewer faults to go undiagnosed (i.e. fewer timeouts) when [PI] help was available. These are positive indications that [PI] acts so as to make complex faults easier to detect and diagnose.

Phase 2 of the study demonstrated a beneficial effect of [PI] and training in reducing anomaly troubleshooting time. Questionnaires showed that most subjects preferred monitoring the [PI] indicator lights *while* monitoring waveforms, rather than monitoring the waveforms alone. On one hand, [PI] did not improve the reliability of detection, since subjects were not any more correct in their anomaly detection with [PI] than without it. On the other hand [PI] did even out performance by reducing the chance of an undiagnosed fault, and by helping subjects with different tasks based on their experience level. It was shown that [PI]'s indicator lights only needed to be 40% reliable for subjects to achieve optimum performance, which shows its flexibility. [PI] correctly detected the anomalous signal for up to 85% of the time. There was no difference in fault management performance between genders.

Implications of Results

Our space experiences with computer decision aids for astronaut scientists have all been demonstrations, rather than formal experiments with testable hypotheses. The drive to develop useable new technology in feasible, cost-effective ways outweighed the scientific need to fly placebo devices as controls for experiments. (These devices would have contributed nothing to the ongoing experiments, would have consumed valuable space resources, and so were considered unessential/dispensable.) Our study performed thorough ground tests to evaluate the efficacy of our expert system for assisting astronauts in the Space Station era. The diagnostic aids, experimental scheduler, and interesting data monitor were shown to be beneficial for carrying out space experiments. Each of the tools developed throughout the history of [PI] – from STS-40 and STS-58 through STS-90 and STS-95 - can be applied to experiments aboard ISS. Autonomous systems are already being implemented in the ISS, and having a software with embedded knowledge such as [PI] will ensure the scientific and operational success of a mission. These developments could reduce the chance of error caused by human-system interface problems, a concern outlined in section 6.09 of NASA's Critical Path Roadmap.

The development of [PI] can be applied to earth-based domains too. Subjects could be helped by an intelligent fault management system for diagnostics and repairs. Earth-based space research includes projects such as the BIOPLEX. Autonomous fault management systems are already

being used for this testbed of life support systems. The results of human behavior in a fault management situation, such as in this ground study, could lead to better designs for the interface of such systems. Other earth-based applications include home sleep monitoring. Patients or caregivers who are not familiar with sleep instrumentation can use a diagnostic engine to help them detect and repair failures, without data being lost. Therefore, the concept of embedding the knowledge in an autonomous system in the spirit of [PI] can benefit technology on earth.

NSBRI RESEARCH PROGRAM
IMMUNOLOGY, INFECTION AND HEMATOLOGY

Team Leader: Shearer, W. Baylor

Shearer, W. T.	**PI**	**Baylor**	**Space Flight Immunodeficiency**	**53**
Dinges, D. F.	CO-I	Penn		
Kanwar, S.	CO-I	Baylor		
Lee, B. N.	CO-I	M. D. Anderson		
Lugg, D. J.	CO-I	Australia		
Mullington, J. M.	CO-I	Harvard		
Ochs, H. D.	CO-I	Washington		
Pierson, D. L.	CO-I	NASA JSC		
Reuben, J. M.	CO-I	M. D. Anderson		
Rosenblatt, H. M.	CO-I	Baylor		
Sams, C.	CO-I	NASA JSC		
Smith, C. W.	CO-I	Baylor		
Smith, E. O.	CO-I	Baylor		
Smolen, J. E.	CO-I	Baylor		
Butel, J. S.	**PI**	**Baylor**	**Immune Function and Reactivation of Latent Viruses**	**57**
Conner, M. E.	CO-I	Baylor		
Keitel, W. A.	CO-I	Baylor		
Larina, I.	CO-I	Russia		
Lednicky, J. A.	CO-I	Baylor		
Ling, P. D.	CO-I	Baylor		
Lugg, D. J.	CO-I	Australia		
Pierson, D. L.	CO-I	NASA JSC		
Fox, G. E.	**PI**	**U of Houston**	**DNA Probe Design for Preflight and Inflight Microbial Monitoring**	**59**
Willson, R. C.	CO-I	U of Houston		
Alfrey, C. P.	**PI**	**Baylor**	**Neocytolysis: Mechanisms and Limitations**	**62**
Byrne, B. J.	CO-I	University of Florida		
Rice, L.	CO-I	Baylor		
Trial, J.	CO-I	Baylor		

IMMUNOLOGY, INFECTION AND HEMATOLOGY TEAM PROGRAM EXECUTIVE SUMMARY

1. Strengths and Synergy of Team's Research Program

Two of the four National Space Biomedical Research Institute (NSBRI) Immunology, Infection, and Hematology team projects (Space Flight Immunodeficiency, W.T. Shearer, Principal Investigator; Immune Function and Reactivation of Latent Viruses, J.S. Butel, Principal Investigator) are reinforcing and synergistic, in that they both address the same Critical Research Path risks (Immunodeficiency/Infections and Carcinogenesis), and they have actively collaborated in these efforts. These Critical Research Path risks carry a Rank 1 (high priority) and Type III (problem suspected), and these risks have been addressed by both projects. Related to the Shearer and Butel projects is that of G.E. Fox (DNA Probe Design for Pre-Flight and In-Flight Microbial Monitoring) that addresses Critical Research Path Risk of Altered Host-Microbial Interactions (Rank 3, Type III). The purpose of this project is aimed at developing a countermeasures program that will detect bacterial contamination of the spaceship (principally water supply) before it causes infection in astronauts. Somewhat related to the other three projects is that of C.P. Alfrey (Neocytolysis: Mechanisms and Limitations), which examined the possible erythrocyte rupture mechanism as an explanation for the apparent anemia experienced by astronauts as they pass through the stress/shearing force of acceleration and deceleration in leaving and returning to Earth (Critical Research Path Risk of Altered Hemodynamics: Dynamics from Altered Blood Components – Rank 1, Type III).

A real team approach and synergetic results can be easily seen by the efforts of the first two of these four projects (Shearer and Butel). In a sense, these projects are the mirror images of each other. The Shearer project has focused on immune alterations in humans exposed to certain conditions of space travel (stress, isolation, containment, sleep-deprivation, microbial contamination), whereas the Butel project has targeted the identification and quantitation of microbial organisms (principally viruses) in humans exposed to most of the same conditions. In fact, the same subject cohort was used in some of the joint research of these two projects. The common cohorts were two-fold: subjects exposed to the Antarctic winter-over (Dr. Desmond Lugg, Australian National Antarctic Research Expedition [ANARE]) and capsule containment (Dr. Irina Larina, Russian Institute for Biomedical Problems). These two human experiences are thought to mimic some of those that astronauts experience in long-term space travel. Blood, urine, and saliva specimens were obtained from these subjects and initially brought to the National Aeronautics and Space Administration Johnson Space Center in Houston (Dr. Duane Pierson), and then subsequently to the laboratory of Dr. Butel, and finally to Dr. Shearer's laboratory. Plasma specimens for bacteriophage antibodies were sent to the laboratory of Dr. Hans Ochs at the University of Washington in Seattle. In a pre-agreed fashion, specimens were parceled out to each laboratory, where specialized tests of immune function or viral detection and quantitation were performed or planned for the future with stored specimens. The overall plan included provisions for assessing lymphocyte proliferative responses and cytokine production in subjects experiencing reactivation of Epstein-Barr virus, as documented by virus shedding in the saliva and/or DNA probe quantitation of white blood cell pellets. Thus, information on this virus infection in humans exposed to the Antarctic winter or Moscow capsule could be magnified by the team effort of the Butel and Shearer laboratories.

Both Dr. Shearer and Dr. Butel have established synergy projects with other NSBRI investigators involved in sleep deprivation (Dr. David F. Dinges and Dr. Janet M. Mullington – Human Performance, Chronobiology, and Sleep Team). These joint projects were designed to test

another condition of space flight (sleep-deprivation) upon the human immune system and its ability to contain viral infections. Studies with Dr. Dinges and Dr. Mullington focused on the production of sleep-inducing cytokines and their receptors and the impact that these altered levels of messengers of the neuroendocrine-immune system might have on host defense. One such consequence would be the upregulation of viral receptors by elevated TH2-type inflammatory cytokines. An example would be the upregulation of CD21, the B-cell receptor for Epstein-Barr virus by high blood levels of interleukin-10. Studies with Dr. Mullington are looking at the shedding of Epstein-Barr virus and JCV, BKV, and SV40 by humans partially deprived of sleep. Additional total sleep-deprivation studies are planned in grant applications to non-NSBRI sources, including the National Institutes of Health ("Earth-Based Research Relevant to the Space Environment", PA-00-088).

Drs. Shearer and Butel have a long-term collaboration in other scientific areas of investigation (e.g., National Institutes of Health Center for AIDS Research – Butel, Director; Shearer, Co-Director). The NSBRI research support has greatly facilitated these interactive project collaborations, because it has expanded the critical mass of investigators, particularly young investigators, in the science of space biomedicine. Drs. Shearer and Butel will be continuing their collaboration in the NSBRI in the next funding cycle, where they will examine an extremely important condition of space flight -- radiation using a murine model infected with latent viruses. For this purpose, they will be collaborating with members of the NSBRI Radiation Safety Team and investigators (Dr. Daila Gridley and Dr. Gregory Nelson) at Loma Linda University, where a linear accelerator is available for generation of the most prominent form of space radiation – proton radiation, estimated to be 2 Gy in Mars-bound space travelers. These plans will directly address the Critical Research Path risk of carcinogenesis.

In terms of the team collaboration with the two other team projects (those of Fox and Alfrey), there has been more limited interaction. The Fox project has been extremely limited due to a very restricted budget, as recommended by the initial peer review process 3 years ago, and the Alfrey project has been more isolated because of a different emphasis (i.e., cytolysis of new red blood cells). Nevertheless, all four projects have been assisted by the monthly business meeting attendance, conjoint science presentations at national space meetings, and participation in the NSBRI Team Workshop and Retreat of 1999 and 2000 that produced a needs assessment for the NSBRI Request for Applications (NSBRI 00-02): themes and specific research questions. Thus, although two of the team projects did not interact as closely as the other two, there was a genuine team effort in maintaining team integrity, spirit, and overall productivity.

2. Critical Evaluation of Level of Risk Reduction in Team Research Area

As a team, the investigators have made significant progress in performing biomedical research at a basic level that validates the early hypotheses sufficiently, so as to justify proceeding with the next 3-year cycle of NSBRI funding. In the case of the Shearer and Butel projects, these conjoint projects have demonstrated that it is extremely important to move on to another ground-based model of space flight to establish the potential risks of space radiation that might confront astronauts in long-term space travel. These new directions came about when it was discovered that a highly specific assessment of antibody function (response to bacteriophage ϕX-174) was not altered in human subjects during the Antarctic winter-over, arguably the best ground-based model of space flight yet developed. Since this assay tests several aspects of T-cell function, as well as the ability of B-cells to produce antibodies, and because the completion of the assessment of the remainder of T-cell assays (lymphoproliferation to antigens, cytokine production by stimulated lymphocytes, lymphocyte subset distribution) on the remainder Antarctic blood specimens will take up to 1 year to complete, Dr. Shearer and Dr. Butel decided to develop a

murine radiation and latent virus space model with NSBRI Radiation Team collaborators, Dr. Daila Gridley and Dr. Gregory Nelson. In an elegant series of peer-reviewed and published experiments, these two individuals have demonstrated that 3 Gy of either proton or gamma-radiation produced a severe reduction in both T- and B-cells in mice. In collaborative studies, we plan to infect irradiated mice with two latent murine viruses (gammaherpesvirus and polyomavirus) in order to assess the possibility that the latent virus would become activated due to the drop in immunosurveillance power secondary to radiation. Also with these collaborators at Loma Linda University, we plan to attempt development of a countermeasures program with chemical radiation blockers. These experiments will be initiated in the new cycle of NSBRI funding.

In the synergy project of the sleep-deprivation model of space flight, Dr. Shearer and Dr. Dinges clearly have shown that sleep-inducing cytokines and their receptors are elevated in human subjects totally deprived of sleep for 88 hours. Interestingly, subjects deprived of sleep for the same time interval, but permitted two 2-hour naps (12 PM - 2 PM; 12 AM - 2 AM) did not show these changes. Thus, a possible risk reduction was suggested by prescription of short naps in the busy schedules of astronauts manning spaceships going to Mars or in astronauts taking a 1-year duty in the space schedule. Possibly, in a maneuver as simple as enforcing short nap times during the astronauts' busy schedules, the risk of elevation of blood cytokines could be prevented and the risk of reactivation of chronic (latent) viral infections and development of virus-driven malignancy could be reduced.

The planned interaction of Dr. Fox's project with Dr. Butel's might lead to the development of molecular probes that could detect microbial contamination of the spaceship with viruses. This project will require a renewed cycle of funding for both Drs. Butel and Fox, which appears to be a possibility. Preliminary discussions have already been held by these two project leaders for this purpose. Since Dr. Fox's project has an elevated Critical Research Path countermeasures readiness level of 5 (demonstration to prove efficacy), successful collaboration would ensure rapid assessment of its efficacy.

3. Programmatic Implications of Individual Project Results

The accomplishments of the individual research projects of the Immunology, Infection, and Hematology Team have clearly identified two and possibly three strong, interactive project leaders who have demonstrated an excellent ability to work as team members, rather than just as individual project members. Members of the "Space Flight Immunodeficiency" project (Shearer) and "Immune Function and Reactivation of Latent Viruses" project (Butel) have the common goal of trying to determine the Critical Research Path risks to space flight in regard to development of immunodeficiency, infections, and cancer. Dr. Fox's research deals with methodology of microbe (bacteria) detection, somewhat related to the goals of the Shearer and Butel projects. Dr. Alfrey's project has attempted to define the molecular mechanism of space "anemia" (other than the simple explanation of fluid shifts). In the context of the bone marrow pleuripotent stem cell that can differentiate into all blood cells (lymphocytes, neutrophils, erythrocytes, etc.), this project is related to the others. The Immunology, Infection, and Hematology Program identified bone marrow stem cell biology as one of its eight themes for the recompetition of NSBRI funding. Thus, research directed at the impact of space flight conditions upon the total development of the bone marrow stem cell would fit very well into the programmatic theme of the team. In that sense, all of the initial projects of the program contributed to a unified concept. Clearly, the future projects of the program should more closely focus on the central theme of host defense in space travel: detection of alterations in host defense using ground-based space models, identification and quantitation of the types of microbial

infections and cancer that take advantage of a compromised immune system, and identification of the earliest stage of stem cell development where space-induced damage occurs. Incumbent upon these discoveries will be the development of suitable countermeasures that will prevent these injuries from happening. Indeed, there are several powerful countermeasures currently available in clinical immunology, infectious diseases, and hematology/oncology that might lend themselves for this purpose. Replacement of antibody immunity by passive immunization (intravenous immunoglobulin), employment of microbe-specific pharmaceutical agents, and autologous stem cell transplantation could be adapted for space flight if the risks of host defense deficiency, chronic infections, and premature malignancies are shown to be unacceptably high.

RESEARCH AREA:	Immunology, Infection and Hematology
PRINCIPAL INVESTIGATOR:	William T. Shearer, M.D., Ph.D.
ORGANIZATION:	Baylor College of Medicine
PROJECT TITLE:	Space Flight Immunodeficiency
FUNDING:	$165,001 (FY 1998); $365,222 (FY 1999); $490,081 (FY 2000)
TOTAL FUNDING:	$1,020,304

PROJECT EXECUTIVE SUMMARY

1. Key Findings and Discoveries

1.1 Antarctic Winter-Over Model of Space Flight

In collaboration with Dr. Desmond J. Lugg, Head of Polar Medicine, Australian Antarctic Division, Australian National Antarctic Research Expedition (ANARE), we have performed the first assessment of human specific antibody response to a T-cell dependent neoantigen (i.e., bacteriophage ϕX-174) in a space flight model. Previous investigations of humans in space flight or in models of space flight measured only serum immunoglobulin concentrations that do not equate to antibody. The bacteriophage ϕX-174 vaccine is known as the gold standard for evaluating deficiencies of humoral (antibody) immune responses. Therefore, this study was particularly important in quantitating several aspects of human immune responses (e.g., viral clearance, primary [IgM] antibody, secondary [IgG] antibody, and helper T-cell-induced immunologic switching of IgM to IgG production). The conditions of the Antarctic winter-over include stress, isolation, containment, and microbial contamination, which are common to those of space flight but lack the conditions of microgravity and solar radiation. Nevertheless, the Antarctic model is one of the best ground-based models of space flight. With the collaboration of ANARE, we studied test subjects stationed at the Casey outpost in Antarctica and control subjects stationed on Macquarie Island. Macquarie Island subjects are considered suitable controls because they have access to the mainland during the winter, in contrast to those subjects at Casey. In the third month of the Antarctic winter-over, subjects were immunized with bacteriophage ϕX-174 and 6 weeks later were given a booster injection. At Casey, the test subjects' immune responses were equivalent to those of the control subjects on Macquarie Island and to the absolute control data in the testing laboratory of Dr. Hans Ochs at the University of Washington, Seattle. These humoral immune responses do not show the deficiencies of some cellular immune responses (delayed type hypersensitivity [DTH] skin test responses to recall antigens) observed by Dr. Lugg in previous ANARE expeditions. These data are important because they clearly define normal antibody responses to a neoantigen (bacteriophage ϕX-174) during the rigorous conditions of the Antarctic winter-over. Also, they clearly do NOT predict similar normal humoral immune responses in space flight because of the important lack of the space factors of microgravity and solar radiation (protons and gamma-rays). These results dictate the application of new models for the assessment of risks of space flight for producing antibody deficiencies. The animal model of irradiated mice challenged with vaccines or virus challenge would be one such example that will be explored in the next funding extension of the present grant. The Antarctic winter-over experiments will be featured in the January 2001 issue of the well-respected *Journal of Allergy and Clinical Immunology* that will introduce space flight immunology research to its readership, along with a cover illustration of space exploration and a National Space Biomedical Research Institute (NSBRI) videotape of the conditions of space flight and model systems utilized in the publication.

1.2 Sleep Deprivation Model of Space Flight

With the recommendation of the NSBRI External Advisory Council, we began a synergy project collaboration with another NSBRI Team (Human Performance, Chronobiology and Sleep) and Dr. David F. Dinges, Professor of Experimental Psychiatry, University of Pennsylvania. Dr. Dinges had previously demonstrated alterations in certain immune cells and cytokines in human subjects deprived of sleep for 64 hours. The loss of sleep in space flight is a major problem for astronauts, as judged by the fact that the most commonly prescribed medication is sleeping pills. In the synergy project, we measured plasma cytokines in a cohort of human subjects with total sleep deprivation (TSD) or partial sleep deprivation (PSD) for 88 hours. Two sleep regulatory cytokines/cytokine receptors (soluble tumor necrosis factor-alpha receptor 1 [sTNF-αRI] and interleukin 6 [IL-6]) were shown to become elevated in the TSD subjects compared to the PSD subjects. The same two messenger molecules of the neuroendocrine-immune system have been shown to regulate sleep in small animal studies involving instillation in the cerebral cortex. The fact that the PSD subjects did not demonstrate an increase in sTNF-αRI and IL-6, suggests that the short naps interspersed throughout the sleep deprivation period might serve as the basis for a countermeasures approach to the problems of loss of cognitive and mechanical ability in sleep-deprived humans. These exciting results have lead to plans for new synergy projects with Dr. Dinges (now Co-Leader of the NSBRI Neurobiology and Psychosocial Factors Team), involving assessment of upregulation of target cell virus receptors by alterations in peripheral blood cytokines and chemokines. The results of the first study will be co-featured with those of the Antarctica study in the January 2001 issue of the *Journal of Allergy and Clinical Immunology*.

1.3 Anti-Orthostatic Suspension Murine Model of Space Flight

Dr. Wayne Smith and colleagues have made several important observations on acquired and innate immune responses using the anti-orthostatic suppression (AOS) mouse system, which mimics known effects of space flight (i.e., underuse of lower extremities, overuse of upper extremities, limited excursion, containment, and cephalad (head) fluid shift. Dr. Smith was able to show that the AOS mouse produced greater DTH skin test responses to a recall antigen than control mice (orthostatic suppression or untethered). Although these findings may be somewhat confounded by the cephalad fluid shift, they suggest that AOS suppression may accentuate hypersensitivity reactions as part of an induced imbalance in immune homeostasis. It is possible that these data indicate a need to assess by additional model studies the development of autoimmune disease in astronauts in long-term space flight.

In addition, Dr. Smith has demonstrated that endotoxin challenge of AOS mice induced a greater expression of the intracellular adhesion molecule type-1 (ICAM-1) in muscle tissue deprived of fluid. This situation is likely to lead to an influx of leukocytes by virtue of their attraction by ICAM-1 molecules on endothelial cell layers. The muscle atrophy observed in astronauts might be related in part to a possible reperfusion injury due to leukocyte infiltration of blood-deprived muscle tissue.

Finally, as a follow-on study of the conflicting studies in the space research literature where phagocytosis and microbicidal activity of neutrophils were examined in the AOS mouse, Dr. Smith was unable to demonstrate any clear effects of AOS upon oxidative function of peritoneal neutrophils. This study will be published in *Aviation, Space, and Environmental Medicine*.

2. Satisfaction of Hypotheses, Objectives, Specific Aims of Original Proposal

Specific Aim 1 had two objectives: 1) Evaluate ANARE subjects for evidence of immunodeficiency, and 2) Evaluate Johnson Space Center (JSC) capsule-isolated astronauts-in-training for evidence of immunodeficiency. Only the first objective was carried out, because the

JSC capsule studies were postponed by the National Aeronautics and Space Administration (NASA). Of the studies proposed on ANARE subjects, we have completed the assessment of specific antibody function, but we are now assessing cellular immune responses: lymphocyte subsets, mitogen and antigen-induced lymphoproliferation, and cytokine production. The 1999 ANARE specimens of frozen plasma and cells reached us in January - March of 2000 (3 separate shipments), so most of the cellular studies are in progress. Also, critical to the evaluation of cellular immunity is the viability of the cell specimens. Regardless of viability, it will be possible to measure cytokine expression by DNA/RNA technology in the cells.

Specific Aim 1 was modified by inclusion of sleep deprivation experiments in the synergy collaboration project described above. Both the Antarctic and sleep-deprivation studies were sustained by the same hypothesis, namely that conditions of space flight models on Earth would induce alterations in the human immune system that might indicate the need to anticipate defects of immunity developing in astronauts on long space voyages (e.g., 3-year trip to Mars). In the first objective, the Antarctic antibody studies did not validate the hypothesis, but they pointed to the need to change the model so that space factors not present in the Antarctic winter-over model could be examined. Thus, in the new grant cycle, we will use irradiated mice challenged by latent virus (gammaherpesvirus and polyomavirus), in collaboration with Dr. Daila Gridley and Dr. Gregory Nelson, radiation biologists at Loma Linda University. The modified objective of the first specific aim (sleep- deprivation studies) did validate the hypothesis, because it showed that sleep deprivation of 88 hours induced significant alterations in cytokine messenger molecules that connect the neuroendocrine-immune system. These sleep deprivation-induced changes in TNF-αRI and IL-6 led to the development of the next hypothesis: altered chemokine levels in astronauts or in ground models of space flight will upregulate viral receptors on target cells and lead to chronic infection and possibly development of malignant clones of transformed lymphocytes, such as Epstein-Barr virus (EBV)-driven lymphomas. This new hypothesis will be tested in ongoing synergy projects, in collaboration with Dr. David Dinges.

Specific Aim 2 of the original project had several components, but the basic objective was to determine whether biochemical or structural components of the inflammatory system and cellular molecule adhesion system are altered in the AOS murine model of space flight. The underlying hypothesis was that local tissue fluid shifts associated with the AOS model would affect the tissue distribution of adhesion molecules and, therefore, alter inflammatory responses. This hypothesis was partially validated in the experiments to date, in that an enhanced DTH skin test response to a recall antigen was seen in the AOS mouse, and an upregulation of endotoxin-induced ICAM-1 molecules was documented in fluid-deprived muscle tissue. These results indicate a need to pursue the important research area of muscle reperfusion, since it is well known that astronauts suffer from muscle atrophy.

3. Implications of Project Research for Risk Reduction in Critical Research Path

The Critical Research Path Risks addressed by Specific Aims 1 and 2 in this research project were: 1) immunodeficiencies/infections (rank 1 [high priority], type III [problem suspected]) and 2) carcinogenesis (rank 1, type III). Thus, we intended to demonstrate whether there should be concern for the possible harmful effects of known conditions of long-term space flight. Our progress to date leads us to believe that continuation of this plan is very important for the preparation for interplanetary space travel. The unknown effects of microgravity and solar radiation (estimated 2 Gy) upon the normal balance of immunity needs careful exploration with additional models of space flight. There is ample clinical evidence from patients on Earth that the immunosurveillance system, when suppressed by virus infection (e.g., AIDS), by therapeutics (e.g., corticosteroids, immunomodulators, cytotoxic agents), by radiation exposure, and by

unremittant stress (e.g., family caregivers to patients with terminal diseases such as Alzheimers) begins to fail in protecting the host against opportunistic infections (e.g., Pneumocystis carinii pneumonia), reactivation of latent viral infections (e.g., EBV infection), and malignancies (e.g., lymphomas, leukemias). The purpose of pursuing these potential developments in astronauts subjected to the potential dangers of space flight is to first examine their feasibility, and to then devise a countermeasures program to negate their impact on astronauts. Currently, our countermeasures readiness level is 1 or 2 (basic research level), but our sleep-deprivation experiments have already suggested that short intermittent naps (2-hour naps every 12 hours) prevent the increased production of the neuroendocrine-immune system messengers involved in sleep regulation. This, at least in a preliminary sense, is a significant beginning to the development of a countermeasure for the immune consequences (infections, cancer) of sleep-deprivation in the long-term perspective. These findings also hold importance for a countermeasures program for diseases and conditions with immediate consequences on Earth (e.g., sleep-starved workers, truck drivers, and pilots who sustain accidents). The experiments with the AOS mouse model that demonstrated an upregulation of ICAM-1 molecules in fluid-deprived muscle tissue possibly may related to the muscle atrophy observed in astronauts. It is well known that in reperfused cardiac tissue after heart attacks, there is an upregulation of ICAM-1 molecules that leads to destructive infiltration of leukocytes. Possibly, the same mechanism operates in space-flight muscle atrophy as part of the muscle disuse mechanism.

RESEARCH AREA:	**Immunology, Infection and Hematology**
PRINCIPAL INVESTIGATOR:	**Janet S. Butel, Ph.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Immune Function and Reactivation of Latent Viruses**
FUNDING:	**$277,000 (FY 1998); $283,648 (FY 1999); $333,648 (FY 2000)**
TOTAL FUNDING:	**$894,296**

PROJECT EXECUTIVE SUMMARY

A major concern associated with long-duration space flight is the possibility of infectious diseases posing an unacceptable medical risk to crew members. One major hypothesis addressed in this project was that space flight will cause alterations in the immune system that will allow latent viruses that are endogenous in the human population to reactivate and shed to higher levels than normal, which may affect the health of crew members. The second major hypothesis examined was that the effects of space flight will alter the mucosal immune system, the first line of defense against many microbial infections, including herpesviruses, polyomaviruses, and gastroenteritis viruses, rendering crew members more susceptible to virus infections across the mucosa.

We focused the virus studies on the human herpesviruses and polyomaviruses, important pathogens known to establish latent infections in most of the human population. Both primary infection and reactivation from latent infection with these groups of viruses (especially certain herpesviruses) can cause a variety of illnesses that result in morbidity and, occasionally, mortality. Both herpesviruses and polyomaviruses have been associated with human cancer. Whereas normal individuals display minimal consequences from latent viral infections, events which alter immune function (such as immunosuppressive therapy following solid organ transplantation) are known to increase the risk of complications as a result of viral reactivations. As this was a new research effort for the project team, initial efforts included development of dedicated laboratory facilities, training of personnel, establishment of collaborations, and development of assays. Special cages were tested, design modified, and then fabricated for the antiorthostatic suspension (AOS) mouse mucosal immunity studies.

The strategy of this project was to measure the frequency and magnitude of viral shedding from humans participating in activities that serve as ground-based models of space flight conditions. First, however, using sensitive polymerase chain reaction (PCR)-based assays for herpesviruses and for polyomaviruses, we established baseline patterns of virus reactivation and shedding in normal healthy volunteers (n = 30) in a one-year-long longitudinal study. We found that normal individuals over age 40 frequently shed polyomavirus JCV in urine, and some normal individuals shed high levels of herpesvirus EBV in saliva, indicating that viral contamination within a spacecraft is an issue to be considered. We then organized collaborations involving several ground-based human models that mimic certain aspects of space flight. These included wintering-over in Antarctica (collaborators D.J. Lugg and D.L. Pierson); a Russian closed chamber study in which individuals were confined within a space-craft-like chamber on the ground (collaborator I. Larina); a sleep-disruption model (collaborator, J. Mullington); and HIV-infected individuals, a medical condition in which patients suffer immunosuppression due to infection with HIV, the AIDS virus (collaborator, C. O'Sullivan). Analyses of specimens from these space analog models are still in progress, but there is preliminary evidence of increased viral reactivation and shedding, suggesting that space flight conditions can alter the host-

pathogen status and result in viral reactivation. These types of data will guide decision-making regarding the necessity of countermeasure development.

We addressed the mucosal immune system questions by using a ground-based mouse model (AOS of mice) and rotavirus (a gastroenteritis virus known to be a mucosal immunogen and to cause human disease). This model system does not simulate all aspects of space flight, but it is accepted as a model for studies on alterations of the immune system. Our results from the AOS mouse model suggest that alterations in mucosal immune responses do occur under simulated space flight conditions, but that neither a delay in rotavirus clearance nor possible alteration of IgG1 anamnestic antibody responses was critical for the resolution of primary rotavirus infection or protection from rotavirus challenge. However, our experiments do not exclude that other important alterations in the mucosal immune system may have occurred. We believe that an examination of more global changes in the mucosal immune system would provide a more thorough cataloging of the effects of AOS on the mucosal immune system.

RESEARCH AREA:	**Immunology, Infection and Hematology**
PRINCIPAL INVESTIGATOR:	**George E. Fox, Ph.D.**
ORGANIZATION:	**University of Houston**
PROJECT TITLE:	**DNA Probe Design for Preflight and Inflight Microbial Monitoring**
FUNDING:	**$48,000 (FY 1998); $49,152 (FY 1999); $49,152 (FY 2000)**
TOTAL FUNDING:	**$146,304**

PROJECT EXECUTIVE SUMMARY

Crew health is a dominant issue in manned space flight. Microbiological concerns, in particular, have repeatedly emerged as determinants of flight readiness. Microbial infection is prominently featured as a possible risk in the Critical Path Roadmap document, and means of monitoring the microbial environment are an important class of countermeasures to be developed. It is essential to the success of long-term missions that systems that deliver acceptable quality of air and water during the anticipated lifetime of the spacecraft be available. As mission duration and re-supply intervals increase, it will be necessary to rely on advanced life support systems which incorporate both biological and physical-chemical recycling methods for air and water as well as provide food for the crew. It therefore is necessary to develop real-time, robust, in-flight monitoring procedures. It would also be desirable if the monitoring system could be readily "reprogrammed" to identify specific pathogens if an in-flight incident were to occur. Thus, the monitoring technology must simultaneously detect many organisms of interest, be subject to miniaturization and be highly automated. The long-range goal of the project was to develop such monitoring systems. In the shorter term, it would be possible to use the technology being developed to obtain a better understanding of the effects of the space environment on microorganisms

Our underlying hypothesis was that the most appropriate target is either ribosomal RNA or the DNA that encodes it. The small subunit rRNA sequence (16S rRNA) in particular has been determined in several thousand bacterial species. Each of these sequences contains short sub-sequences that are widely conserved throughout the data set as well as other sub-sequences totally unique to, and characteristic of, a particular species. This pattern of sequence conservation made it possible to design oligonucleotide hybridization probes that can distinguish individual organisms, or groupings of organisms. Once an appropriate set of target sub-sequences have been identified for a desired assay, any of a variety of formats can be used to implement the assays. Thus, the final assay system may utilize PCR-amplified nucleic acids or, because rRNAs are high copy number molecules, direct detection systems such as chemiluminescence or fluorescence. Both types of assay are compatible with miniaturization and data can be processed automatically or returned to Earth by telemetry. The project objectives included an examination of alternative implementations and development of space craft compatible methods for sample processing.

The major Project Accomplishments were as follows:

1. We initially demonstrated the feasibility of a DNA chip based assay for monitoring of water quality using probes that target 16S rRNA. This result also validated our probe designs for several species of spaceflight interest. It was at the same time clear that three major issues needed to be addressed to make the approach truly useful. These are (1) development of an appropriate set of hybridization probes with minimal cross-reactivity, (2) the development of spacecraft compatible procedures for extracting and purifying the target nucleic acids and (3)

increasing the sensitivity and ease of execution of the assay. The work undertaken in subsequent years focused on these issues and considerable progress was made towards resolving them. This progress was achieved in part through the development of advanced software for probe design, and also through other accomplishments described below.

2. We established specific probes for essentially all the organisms needed to devise an assay system for monitoring spacecraft water quality. This included probes for total bacteria, Gram negative bacteria, enteric bacteria, *Escherichia coli*, *Vibrio proteolyticus*, *Burkholderia cepacia*, and *Acinetobacter*. Several of these probes will also be required for an air analysis system. Several of these probes have now been successfully utilized in multiple assay formats, including molecular beacons as discussed below.

3. We developed compaction precipitation for purifying DNA and RNA. The new technique, which has significant Earthbound spin-off potential, will be particularly useful in developing and possibly in performing spacecraft-based nucleic acid probe assays. A patent application has been filed, and a *Nature Biotechnology* article on the technique generated a high level of inquiries from outside laboratories. The method is being utilized in research on plasmid-based DNA vaccines for HIV, and the email protocols we have sent out appear to be spreading from user to user. The method has the potential for broad use in molecular biology for cloning, sub-cloning, genomics, DNA sequencing, etc. UH has identified a likely licensee for the technology, and as licensing terms are being finalized plasmid miniprep kit design has advanced to the point that the licensee now has packaging mockups for the commercial spin off product.

4. We found that a well-known method of protein purification is also very effective for many nucleic acid separations. Immobilized-metal affinity chromatography (IMAC) is the basis of the ubiquitous six-histidine purification "tag" for recombinant proteins. We hypothesized that chelated metals might also form ligand interactions with the exposed aromatic base nitrogens of single-stranded nucleic acid molecules. Surprisingly, this prospect has not been previously investigated. IMAC proves to be extremely effective at capturing RNA from mixtures with other molecules, and also for stripping primers e.g., from PCR and sequencing reactions. At least some (possibly all) single-base mismatches can be detected, raising the possibility of developing IMAC-based hybridization assays for microbial identification, SNP scoring, etc. A publication and a patent application are in preparation, and the UH licensing office is in negotiation with at least 5 prospective licensees, including the dominant companies in the field.

5. We applied molecular beacons to rapid, low-labor detection of organisms of spaceflight interest. These DNA hairpin probes bear a fluorophore at one end and a quencher at the other. The beacon becomes highly fluorescent when bound to target sequences in an extended configuration. The resulting homogeneous assay also has the advantage of minimal waste generation and reduced danger of cross-contamination, especially when used with amplification methods such as PCR or NASBA. We have converted several probes for organisms of space flight interest into beacon formats, and demonstrated simultaneous multiplex detection of several organisms using fluors with non-overlapping spectral properties ("colors"). In preliminary results toward highly parallel detection we have also demonstrated the feasibility of arrays of immobilized beacons, in which positive signals are identified by the position, rather than the color, of fluorescence emission.

These results have significant implications for future work. At this stage enough progress has been made in probe design that it would be possible to begin actual instrument development. This should, however, be accompanied by further refinement and testing of the already validated probes. Additional probes can also be designed and validated such that a prototype instrument could examine either air or water samples. Probe design can also now focus on the identification of possible pathogens or otherwise problematic bacteria without preconceived notions of what these organisms will be.

The two main methods under consideration for microbial monitoring are array hybridization and molecular beacon technology. Both are attractive approaches because they might also be usable with samples from blood, urine, and other crew-derived specimens, as well as water and bio-regenerative life-support system samples. This possibility has been further facilitated by the development of space-craft compatible methods for handling samples. Finally, if a hybridization array instrument were developed for microbial monitoring it would also be useable for in-flight studies of global gene expression. This approach might be an excellent way to determine whether key properties such as growth rates, mutation rates or pathogenicity are likely to be affected by the space environment.

RESEARCH AREA:	**Immunology, Infection and Hematology**
PRINCIPAL INVESTIGATOR:	**Clarence P. Alfrey, M.D., Ph.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Neocytolysis: Mechanisms and Limitations**
FUNDING:	**$230,000 (FY 1998); $235,520 (FY 1999); $117,670 (FY 2000)**
TOTAL FUNDING:	**$583,190**

PROJECT EXECUTIVE SUMMARY

Astronauts who go up into space have too much blood for their new environment. The hormone erythropoietin, which controls the production of red blood cells, quickly becomes suppressed. Our data from spaceflight demonstrates that there ensues destruction of young red blood cells less than 12 days old, allowing rapid adaptation. On re-entry, astronauts find themselves maladapted for function in a gravitational environment, being hypovolemic and anemic. To confirm our theory of neocytolysis, we studied high altitude residents who descend to sea level. Like astronauts entering microgravity they suddenly find themselves with excessive blood mass for their new environment. As predicted, erythropoietin levels fell and the number of red blood cells fell very quickly. Destruction of the red cells could be prevented by low doses of erythropoietin.

In our current studies, we are dissecting the mechanisms effecting neocytolysis, the selective destruction of young red blood cells. We have been able to demonstrate erythropoietin receptors exist on splenic endothelial cells. This is important because the spleen is the most likely site of neocytolysis. We have created an *in vitro* model of the process in which endothelial cells grow above macrophages. When the cells are grown in erythropoietin-containing medium and then have erythropoietin withdrawn, splenic endothelial cells become more permeable. They allow increased diffusion of large sugars and they allow increased phagocytosis of young RBCs by macrophages. Young red blood cells seem to be preferential targets in this model, as *in vivo*. Interestingly, endothelial cells from human aorta, umbilical veins or renal glomeruli do not respond to erythropoietin withdrawal in the manner of splenic endothelial cells. This *in vitro* model continues to yield insights on the mechanisms of neocytolysis.

A rodent model of neocytolysis could greatly facilitate our ability to dissect and experimentally manipulate the process. We have been very successful using AAV—viral vectors to deliver the EPO gene to mice. This establishes stable, high expression of the gene leading to marked polycythemia in the animals. We are co-delivering a tetracycline response control gene and we can successfully turn off the EPO gene expression with tetracycline in *in vitro* cultured cell lines. We are actively working on turning off erythropoietin with tetracycline in the polycythemic mice, which should precipitate neocytolysis and establish a model for experimental manipulation.

We have established a human model of neocytolysis by injecting volunteers with erythropoietin, increasing their red cell mass, then withdrawing the erythropoietin. We have observed a rapid fall in the number of red cells on erythropoietin withdrawal, just as predicted. One observation that has emerged is that changes in serum ferritin concentration serve as a precise inverse mirror of the changes in red cell mass. Serum ferritin reflects the amount of iron in body stores. As red cell mass increases under the influence of supplemental erythropoietin, serum ferritin falls as iron is mobilized from stores into newly synthesized hemoglobin. When red cell mass falls, as with neocytolysis, ferritin rises rapidly as iron is transferred back to stores. We have found that

ferritin very precisely reflects the changes in red cell mass in space, in altitude-dwellers descending to sea level and now in this erythropoietin-driven human model. The recognition of the utility of ferritin levels in these situations should simplify our ability to study the process. Ferritin levels could also be used clinically as an early measure of the effectiveness of erythropoietin therapy in human disease.

The rate of change of ferritin concentration is much slower when erythropoietin is augmented than when the erythropoietin is decreased which matches our observations in spaceflight that deadaptation to earth's environment as manifest by decrease in red cells occurs quickly. No adverse reaction occurred in normal volunteers receiving erythropoietin for three to six weeks. The model we have established will permit determination of the minimal erythropoietin dose required to prevent deadaptation.

Our discoveries emanating from the unique environment encountered in space have yielded insights on previously unrecognized physiologic and pathophysiologic conditions on earth. We are hopeful that our studies will encourage effective countermeasures for space travelers re-entering a gravitational environment. Our observations clearly impact on such diverse situations as altitude adaptation and de-adaptation, training of elite athletes, anemia of renal disease, optimal erythropoietin dosing schedules, hemolytic anemia and polycythemias. Continuing unraveling of these phenomena will further demonstrate the unforeseen benefits that accrue when basic problems in space are scientifically addressed.

NSBRI RESEARCH PROGRAM
MUSCLE ALTERATIONS AND ATROPHY

Team Leaders: **Hamilton, S. L.** **Baylor**
Schwartz, R. J. **Baylor**

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Booth, F. W.	CO-I	Univ. of Missouri at Columbia		
Schwartz, R. J.	CO-I	Baylor		

MUSCLE ALTERATIONS AND ATROPHY TEAM PROGRAM EXECUTIVE SUMMARY

A current goal of the National Biological Research Institute is to develop countermeasures that allow humans to live and work in microgravity for durations over a year and to minimize readapting to Earth's gravity, and optimize crew safety, well-being, and performance. Exposure to reduced gravity during space travel profoundly alters the loads placed on muscle. Astronauts lose muscle mass and strength while in space. Their ability to work upon reexposure to gravity is thus reduced. This is a safety concern. Individuals with weaker muscles are less likely to survive an unexpected event requiring muscle strength to save life. Although, a large amount of work has been published assessing the efficacy of various exercise modalities the results of these studies can be summarized as follows: virtually all protocols of exercise and/or muscle loading do not prevent muscle atrophy.

The mission of the Muscle Alteration and Atrophy Team is to foster an investigative and highly interactive research program towards understanding basic mechanisms involved with maintenance of muscle mass and muscle function under conditions of long term microgravity. The most effective countermeasure to atrophy of muscle in space flight is likely to be developed from the mechanism producing the atrophy. Without knowing the fundamental molecular mechanisms underlying muscle atrophy, the development of effective countermeasures to muscle atrophy from unloading is less likely to be an optimal countermeasure. Similar wasting of muscles affects people on earth during prolonged bed rest, immobilization, and nerve crush injury, motor neuron diseases and aging. Likewise, knowledge of the mechanisms of muscle atrophy from unloading is likely to provide more effective countermeasures. Most mechanistic information about the cause of muscle atrophy with unloading will be obtained from animals. This is due to: 1) the work schedules of astronauts prohibiting their involvement as subjects, 2) the inability to collect enough muscle from astronauts, and 3) the future necessity to use transgenic animals inflight. Experiments performed in animals will provide guidelines for design of human experiments and will provide molecular/biochemical markers of muscle atrophy.

The proposed investigations will shed light on the pathogenesis of muscle atrophy and fiber type conversion at a system, cellular, and molecular level, drawing on the Muscle Team's depth of expertise on the neuromuscular junction (NMJ), excitation-contraction coupling (E-C), muscle gene regulation, and trophic interactions. The objectives are to mount effective countermeasures by pharmacological, physiological, and gene-therapeutic means, to blunt muscle atrophy during weightlessness in long term space flight, and to provide important novel spin-back applications to ameliorate related human health problems of muscle wasting on earth. The proposed countermeasures encompass a complementary set of workable short and longer-term interventions including:

- an emphasis on the growth hormone/insulin-like growth factor axis, and its interaction with physical countermeasures (resistive and concentric exercises);
- pharmacological inhibitors of muscle proteolysis;
- injectable gene-based medicines expressing growth hormone releasing hormone;
- fundamental mechanisms coupling muscle excitation and cellular stretch signaling via calcium fluxes, NFAT-calcineurin, myotonic dystrophy kinase to muscle gene expression
- basic helix–loop-helix transcription factors governing muscle fiber type during stimulated microgravity;
- longitudinal assessment of NMJ functions in animals and humans.

RESEARCH AREA:	**Muscle Alterations and Atrophy**
PRINCIPAL INVESTIGATOR:	**Robert J. Schwartz, Ph.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**GH/IGF-I Transgene Expression on Muscle Homeostasis**
FUNDING:	**$239,674 (FY 1998); $232,249 (FY 1999); $232,249 (FY 2000)**
TOTAL FUNDING:	**$704,172**

PROJECT EXECUTIVE SUMMARY

We propose to test the hypothesis that the growth hormone/insulin like growth factor-I axis through autocrine/paracrine mechanisms may provide long-term muscle homeostasis under conditions of prolonged weightlessness. As a key alternative to hormone replacement therapy, ectopic production of hGH, growth hormone releasing hormone (GHRH), and IGF-I will be studied for its potential on muscle mass impact in transgenic mice under simulated microgravity. Expression of either hGH or IGF-I would provide a chronic source of a growth-promoting protein whose biosynthesis or secretion is shut down in space. Muscle expression of the IGF-I transgene has demonstrated about a 20% increase in hind limb muscle mass over control nontransgenic litter mates. These recent experiments, also establish the utility of hind-limb suspension in mice as a workable model to study atrophy in weight bearing muscles. Thus, transgenic mice will be used in hind-limb suspension models to determine the role of GH/IGF-I on maintenance of muscle mass and whether concentric exercises might act in synergy with hormone treatment. As a means to engineer and ensure long-term protein production that would be workable in humans, gene therapy technology will be used by to monitor muscle mass preservation during hind-limb suspension, after direct intramuscular injection of a genetically engineered muscle-specific vector expressing GHRH. Effects of this gene-based therapy will be assessed in both fast twitch (medial gastrocnemius) and slow twitch muscle (soleus). End-points include muscle size, ultrastructure, fiber type, and contractile function, in normal animals, hind limb suspension, and reambulation.

RESEARCH AREA:	**Muscle Alterations and Atrophy**
PRINCIPAL INVESTIGATOR:	**Alfred Goldberg, M.D.**
ORGANIZATION:	**Harvard Medical School**
PROJECT TITLE:	**Pharmacological Inhibitors of the Proteosome in Atrophying Muscles (Also Kenneth Baldwin, Ph.D., UC Irvine, Effects of Unloading on Myosin Content and Isoform Specific Regulation in Skeletal Muscle)**
FUNDING:	**$343,965 (FY 1998); $318,176 (FY 1999); $298,196 (FY 2000)**
TOTAL FUNDING:	**$960,337**

PROJECT EXECUTIVE SUMMARY

Studies of the Ubiquitin-Proteasome Pathway (Dr. Alfred Goldberg)

1) Critical Role of Certain Ubiquitination Enzymes

Various prior observations had suggested that the increased protein breakdown responsible for muscle atrophy in many diseases (e.g. cancer, sepsis, diabetes, or hyperthyroidism) is primarily due to an activation of the Ub-proteasome pathway. During the past three years using homogenates of normal and atrophying muscles, we showed that overall rates of Ub-conjugation increase in atrophying muscles, and these hormone and cytokine-dependent responses are due largely to activation of the N-end rule pathway for Ub-conjugation. Specifically we discovered that Ub-conjugation to endogenous proteins and to a model substrate of the N-end rule pathway (lysozyme) were significantly increased. We found that mRNA for the critical ubiquitination enzymes in the N-end rule pathway, $E2_{14k}$ and E3α, are increased about 2-fold in the atrophying muscles. Thus, the activation of Ub-conjugation and proteolysis by the "N-end" pathway appears to be a very general feature of atrophying muscles, independent of the cause.

In normal muscle extracts, we found that the N-end rule pathway for ubiquitin conjugation also appears to be responsible for the degradation of most soluble proteins. In contrast to muscle, in extracts of HeLa cells, this system is also present but makes only a minor contribution to overall protein ubiquitination. These findings were quite unexpected, because the ubiquitinating enzymes comprising the N-end rule pathway, and in particular E3α, are believed to recognize abnormal proteins with unusual amino-terminal residues. An important role for $E2_{14k}$ and E3α in muscle (or in any other cell) was unanticipated since the synthesis of all proteins begins with a methionine in the N-terminus, and over 80% have their N-termini acetylated, which prevents recognition by the E3α.

2) Ubiquitination Conjugation After Hind-Limb Suspension.

A similar increase in ubiquitin conjugation was found in extracts of muscle following hind-limb suspension (performed by our collaborator, Dr. Ken Baldwin). These findings suggest that the disuse atrophy in astronauts involves a similar activation of the Ub-proteasome pathway, as we have found in many other types of muscle wasting (although these changes upon hind-limb suspension were less pronounced and therefore harder to study routinely than in some other atrophying models).

3) Possible Use of Protease inhibitors

One other goal of our work during the initial grant period was to evaluate the possible utility of the newly discovered proteasome inhibitors as possible countermeasures to retard the excessive

protein breakdown in atrophying muscles. It is noteworthy that these inhibitors, especially the peptide aldehydes, had much larger effects in reducing proteolysis in atrophying muscles than in control muscles. Thus, as suggested previously, the enhanced proteolysis in many catabolic states is due to a proteasome-dependent pathway. Because inhibition of proteasome function in principle could be a useful approach to reduce muscle wasting, we carried out systematic studies to define the effects of peptide aldehydes and the more selective proteasome inhibitor, lactocystin-β-lactone on intracellular proteolysis. These findings provided definitive support for our prior conclusions that the proteasome is the site for degradation of long-lived as well as short-lived proteins. On the other hand, we and a number of other investigators have found that prolonged exposure of most cells to these various inhibitors (i.e. for 24-32 hours) at concentrations that cause a large reduction in protein breakdown causes cell death by apoptosis. So, although partial inhibition of proteasome function is well tolerated, complete inhibition is dangerous, therefore these types of inhibitors are inappropriate for use by astronauts as countermeasures and can only be used against life-threatening illnesses in a hospital setting. In fact, such clinical trials are in progress now in cancer patients. Consequently, future studies will focus on development of inhibitors of ubiquitination enzymes that are specifically important in atrophy (whose inhibition should be non-toxic and affect primarily atrophying muscles).

4) Patterns of Gene Expression in Atrophying Muscles

It seems likely that (1) muscle atrophy involves a suppression of the same program of gene expression that is activated during work-induced hypertrophy or by IGF in normal growth and (2) that there also exist a number of genes induced during muscle atrophy that are of major importance in the loss of muscle protein and contractile function. Our studies thus far have uncovered several genes (e.g. Ub, proteasome subunits, etc.), whose expression increases approximately 2-3 fold in all types of muscle atrophy studied thus far. The increases in these mRNAs are noteworthy because atrophying muscles generally show a decrease in total mRNA and ribosomal RNA. We propose to call such genes, atrophy genes, since these gene products which we call "atrophins" are likely to play a key role in the process of muscle wasting.

Because of the potential value of information on the nature of these atrophy genes, during the past year, under the NSBRI grant we have initiated a gene microarray analysis to obtain a comprehensive picture of the transcriptional changes occurring during muscle atrophy. This approach allows comparison on a single chip of mRNAs from experimental and control tissues from mouse or human cDNA libraries containing 5-10,000 different cDNAs. In order to validate this approach, in our initial experiments we chose to study the pattern of changes in muscle mRNAs induced by fasting, primarily because of the simplicity of this model and the wealth of prior information on this type of muscle wasting, especially the changes in proteolysis and energy metabolism. Our initial observations have proven very informative and promising. We have found over 100 genes whose levels change by about 2-fold or more in fasting. They fall into several categories including mRNAs encoding a) multiple subunits of the 20S proteasome and its 19S regulatory complex, which are coordinately up-regulated (as expected). Most interestingly, there are 7 genes (ORFs) whose expression increases most markedly (4-9 fold), and surprisingly, the functions of all of them are unknown. We are beginning to clone the most highly induced species in order to analyze their expression, to see if they are induced upon unloading, to prepare antibodies against the encoded proteins, and to explore their functions. The protein encoded by this most highly regulated mRNA, which we term atrophin-1, resembles that a subunit of a new type of E3 (a ubiquitination protein ligase) belonging to the F-Box. These exciting observations suggest that this protein is part of a new ubiquitination enzyme involved in the acceleration of protein breakdown during muscle wasting.

Effects of Unloading on Myosin Content and Isoform Specific regulation in Skeletal Muscle (Dr. Kenneth M. Baldwin)

History of the Project

In the initial funding period supported by the NSBRI (October, 1997-September, 2000) our original project entitled "Effects of Unloading on Myosin Content and Isoform Specific Regulation in Skeletal Muscle" was jointly submitted along with a project proposed by Dr. Alfred Goldberg's group at Harvard Medical School (K.M. Baldwin and A. L. Goldberg, serving as Co-PIs). The central thrust of the proposal was to examine how unloading (and increased loading) states impact the expression of the MHC protein system by examining transcriptional, translational, and degradative (ubiquitin-proteasome) processes. Although the project was funded, the NSBRI appointed Dr. Goldberg as the PI and the proposal was given a new title with a primary focus on degradative processes associated with the ubiquitin-proteasome pathway. The Baldwin project had to be de-scoped, since the budget was reduced by >50% thereby precluding performing any studies related to protein synthesis of the MHC isoforms due to insufficient resources. Instead our group focused on a narrower set of objectives: a) regulation of transcription of the slow MHC gene in response to altered loading states; and b) the delineation of myogenic factors in the control of muscle mass and contractile phenotype.

Progress on the Original Proposal

Significant progress was attained on four interrelated projects: 1) *in vivo* regulation of the beta (slow) MHC gene in soleus muscle of suspended and weight-bearing rats; 2) changes in markers of myogenesis in overloaded rat muscles; 3) mechanisms on up regulation of fast IIb MHC in unloaded muscle; role of the nerve and thyroid hormone; and 4) quantitation of total MHC and MHC isoforms in response to unloading.

In the first project we demonstrated the feasibility of using direct DNA transfection technology for studies on the *in vivo* regulation of the type I MHC gene promoter in response to weight bearing activity and hindlimb suspension. In that project, we demonstrated that a) normal (optimal) type I MHC transcriptional activity in antigravity muscles requires the presence of an up-stream enhancer sequence (-3500 to -2900) that likely interacts with response elements in the first 400 bp upstream of the transcription start site (TSS); b) unloading-induced down regulation of type I MHC promoter activity is mediated in the proximal 400 bp upstream of the TSS. [We have tentatively identified the negative beta e1 response element as a key factor in this process]. Additional studies are in progress to more fully characterize the proximal response elements in response to unloading. A paper on this project has been published and is included in appendix A.

The second project was aimed at identifying markers of putative satellite cell proliferation and differentiation processes in muscles that undergo increases in hypertrophy due to increases in chronic loading. These experiments were performed in the context of increased expression of muscle IGF-I at both the mRNA and peptide level. The central findings of this study indicate that myogenic processes are activated in response to increased loading at early time points (e.g. 12 hrs) and that IGF-I is likely modulating this response. Furthermore, the findings indicated that some myogenic cells are likely differentiating early on in the adaptive process, before events leading to satellite cell proliferation have been initiated. Some of the data from this project is presented in the context of the current proposal in the next section. A copy of this paper is provided in Appendix A.

The third project was aimed at understanding how the ~ de novo expression of fast type IIb MHC gene occurs in antigravity muscles, e.g., muscle-types that do not normally express this gene.

This work was predicated on the novel observation that hindlimb unloading requires increased levels of thyroid hormone in order to fully express the IIb MHC gene at both the mRNA and protein levels. Our finding suggest that normal innervation is essential for inducing the unique expression of the IIb MHC in a slow muscle in response to the combination of hindlimb suspension and thyroid hormone; and the up regulation of the myogenic factor, MyoD, may be essential to this process. However, in the denervated muscle, there is a discordance between the regulation of the endogenous IIb MHC gene relative to the exogenous IIb promoter-report construct that is not fully understood at the present time. A copy of the published article on these findings is provided in appendix A.

In the fourth project we developed techniques to quantitate changes in total as well as isoform specific MHC protein and mRNA content in response to unloading in order to show that during unloading, the myofibril system (and particularly the contractile apparatus) undergoes a remodeling in which there are reductions in the slow MHC content at the protein and mRNA levels which accompanies the general degradation process. In addition, there are also maintenance in protein and increase in mRNA content of fast MHCs (IIx-IIb) that occur in spite of the general atrophy process that predominates during unloading. These findings, in conjunction with project III, clearly show that there is MHC isoform-specific gene regulation in response to altered loading states; and these processes are likely mediated by a coordination between transcriptional, translational, and degradation control points. We are in the process of writing a paper on this project, and have provided pertinent data on MHC content summarized in figure 2 the next section.

In summary, we have made significant progress on several fronts in an attempt to address fundamental issues in the biology of muscle plasticity that are relevant to the mission of the NSBRI. However, in view of the fact that future research concerning muscle structure and function funded by the NSBRI needs to be more closely related to seeking countermeasures for reducing muscle atrophy, we have refocused our research to more specifically address the efficacy and mechanisms concerning the role of resistance training in reducing the muscle atrophy that occurs in response to chronic unloading.

RESEARCH AREA:	**Muscle Alterations and Atrophy**
PRINCIPAL INVESTIGATOR:	**Henry F. Epstein, M.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Molecular Signaling in Muscle Plasticity**
FUNDING:	**$109,500 (FY 1998); $112,128 (FY 1999); $112,128 (FY 2000)**
TOTAL FUNDING:	**$333,756**

PROJECT EXECUTIVE SUMMARY

The original specific aims of this project were to:

1. Identify the roles of dystrophin-based pathway as a signaling pathway for muscle plasticity most active in Type II fibers. The *mdx* null mouse line which is dystrophin-negative will be studied.
2. Delineate the roles of the myotonic protein kinase (DMPK)-based system which appears to represent a signaling pathway for muscle plasticity most active in Type I fibers. Knockout and transgenic mouse lines which are either DMPK-negative or overexpressing DMPK will be studied.
3. Characterize the roles of focal adhesion kinase and associated molecules as a general myogenic signaling pathway in muscle plasticity. Transgenic mouse lines with inducible knockout and mutant constructs will be studied.
4. Analyze genetically the interactions between dystrophin, focal adhesion kinase, and DMPK pathways in specific double mutant combinations, and further characterizing the mechanisms by which those pathways regulate muscle plasticity.

We have concentrated our efforts on Specific Aim 2 and closely related aspects of Specific Aims 3 and 4.

The central molecule of interest has been DMPK. The interactions of DMPK with Rac-1 that we have studied are related *in vivo* to the actions of focal adhesion kinase. Rac-1 is a downstream effector of focal adhesion kinase, which is an integral component of the integrin/actin cytoskeleton-signaling pathway that is adhesion-sensitive. The interactions of DMPK with Raf-1 kinase of the chemical signaling pathway and Rac-1 represent an important further characterization of the signaling pathways that regulate muscle plasticity.

In the last year, our work on the UNC-45 myosin assemblase has become relevant to this project because we broadened our focus there from genetic and biochemical experiments in *C. elegans* to the identification and characterization of UNC-45 homologues in humans and mice.

RESEARCH AREA:	**Muscle Alterations and Atrophy**
PRINCIPAL INVESTIGATOR:	**Susan L. Hamilton, Ph.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Activity Dependent Signal Transduction in Skeletal Muscle**
FUNDING:	**$118,152 (FY 1998); $122,177 (FY 1999); $86,619 (FY 2000)**
TOTAL FUNDING:	**$326,948**

PROJECT EXECUTIVE SUMMARY

The overall goals of this project are: 1) to define the initial signal transduction events whereby the removal of gravitational load from antigravity muscles, such as the soleus, triggers muscle atrophy, and 2) to develop countermeasures to prevent this from happening. Our rationale for this approach is that, if countermeasures can be developed to regulate these early events, we could avoid having to deal with the multiple cascades of events that occur downstream from the initial event. One of our major findings is that hind limb suspension causes an early and sustained increase in intracellular Ca^{2+}concentration ($[Ca^{2+}]_i$). In most cells the consequences of changes in $[Ca^{2+}]_i$ depend on the amplitude, frequency and duration of the Ca^{2+} signal and on other factors in the intracellular environment. We propose that muscle remodeling in microgravity represents a change in the balance among several Ca^{2+} regulated signal transduction pathways, in particular those involving the transcription factors NFAT and NFκB and the pro-apoptotic protein BAD. Other Ca^{2+} sensitive pathways involving PKC, ras, rac, and CaM kinase II may also contribute to muscle remodeling.

Mechanisms by which hind limb suspension could alter the concentration of resting Ca^{2+}

In the 02 year of NSBRI funding our goals were to determine both the cause and the functional consequences of the rise in resting Ca^{2+}. Muscle inactivity increases the production of ROS (Hisao *et al*, Am. J. Physiol. 28: E839-E844, 1993) and decreases nitric oxide (NO) production (Tidball *et al*, Am.J. Physiol. 275: C260-C266, 1998). We demonstrated that, in muscle, there is a delicate balance between ROS, NO and calmodulin (CaM) regulation of Ca^{2+} leak via the skeletal muscle Ca^{2+} release channel (RYR1). Both oxidants and Ca^{2+} free CaM activate RYR1, but their interactions with the channel are mutually exclusive. When cytoplasmic Ca^{2+}rises, Ca^{2+}CaM helps to shut the channel, but this effect can be blocked by channel oxidation and, conversely, both Ca^{2+}CaM and Ca^{2+} free CaM partially protect RYR1 from oxidation. NO blocks oxidative activation of RYR1, has no effect on Ca^{2+}CaM inhibition of the channel, but prevents the activation of the channel by Ca^{2+} free CaM. The net effect of conditions that increase ROS but decrease NO would be prolonged opening of RYR1 and increased cytoplasmic Ca^{2+}. These findings have led to our current working hypothesis that the increase in resting Ca^{2+} in skeletal muscle during hind limb suspension arises, at least in part, from increased SR Ca^{2+} leak. Decreases in SR luminal Ca^{2+} could also activate store operated Ca^{2+} influx pathways. Mechanisms to regulate $[Ca^{2+}]_i$ will be investigated in the coming year.

Effects of sustained increases in $[Ca^{2+}]_i$ on muscle function

Increases in intracellular Ca^{2+} can alter a variety of signal transduction pathways and the actual pathways altered will depend on the amplitude, frequency and duration of the Ca^{2+} signal. Our studies show a sustained increased $[Ca^{2+}]_i$ after about 3 days of hind limb suspension. We are exploring the possibility that the increased $[Ca^{2+}]_i$ activates pro-apoptotic pathways leading to

loss of myonuclei and muscle remodeling. Activation of apoptotic pathways has previously been suggested to contribute to hind limb suspension induced muscle remodeling (Allen *et al*, Am. J. Physiology 273: C579-C587, 1997). In B cells low sustained increases in intracellular Ca^{2+} have been shown to activate NFAT while large Ca^{2+} transients activate NFκB and JNK transcription factors (Dolmetsch *et al*, Nature 386:855-858, 1997). For the NFAT pathway, the increases in resting Ca^{2+} activate calcineurin and this dephosphorylates NFAT, allowing it to translocate to the nucleus. The transcriptional events activated by NFAT depend on the status of a number of other signal transduction pathways, but NFAT activation can increase the transcription of several pro-apoptotic genes. We have identified the presence of NFAT in skeletal muscle and have shown that cytoplasmic levels of NFAT decrease with hindlimb suspension. We have not, however, yet shown that this is due to translocation to the nucleus. We are currently assessing the nuclear translocation of NFAT with hind limb suspension.

In addition to its effects on NFAT, calcineurin can dephosphorylate the apoptotic protein BAD (Wang *et al*, Science 284:339-343, 1999), leading to its interaction with the anti-apoptotic proteins Bcl-2 and BClxl. We have detected BAD in skeltal muscle and are currently attempting to assess whether hind limb suspension alters in phosphorylation status. We have, however, shown that there significant increase in cytosolic BAD with 14 days of hind limb suspension. We are currently assessing if this is due to new protein or decreased breakdown. A anti-apoptotic protein that can interact with Bcl-2 is smn (Iwahashi *et al*, Nature 390:413-417, 1997), the protein missing in spinal muscular atrophy. Our preliminary studies suggest an decrease in cytoplasmic smn with hind limb suspension. We also have preliminary evidence for an decrease in cytosolic IκB.

Skeletal muscle specific FKBP12 deficient mice as models for effects of microgravity

FKBP12 is an endogenous modulator of both RYR1 and calcineurin. FKBP12 inhibits calcineurin and stabilizes a closed state of RYR1. The absence of FKBP12 is likely to lead to increases in both resting Ca^{2+} and calcineurin activity. If calcineurin activation produces muscle atrophy, the knockout of FKBP12 may mimic the effects of hind limb suspension on skeletal muscle. An animal model of muscle atrophy would be extremely useful for drug intervention. We have previously prepared FKBP12 deficient mice. Skeletal muscle force production was markedly diminished but, unfortunately these animals die of cardiac hypertrophy. To avoid the cardiac complications we are in the process of creating a skeletal muscle specific knockout of FKBP12 using the Cre-loxP system. To generate the skeletal muscle restricted FKBP12-deficient mice, two transgenic mouse lines will be used, the skeletal muscle specific Cre mouse and the FKBP12-*lox*P mouse. In collaboration with Dr. Weinian Shou, we now have both the FKBP12-*lox*P targeted ES cell lines and the linear myogenin-Cre construct. We anticipate having the skeletal muscle specific FKBP12 knockout mice within the next 6 months. We will compare soleus muscle from these mice to that obtained from hind limb suspended mice to determine if the mechanisms of atrophy are related.

Summary of Progress in 02 year

In the 02 year of NSBRI funding we have demonstrated that: 1) there is an early and sustained increase in intracellular Ca^{2+}, 2) an increase in oxidants or a decrease in NO can increase resting Ca^{2+}, 3) hind limb suspension alters NFκB and possibly NFAT signaling in skeletal muscle, 4) NFκB mediates proteolytic signaling in muscle by upregulating components of the ubiquitin/proteasome pathway, and 5) hind limb suspension increases the amount of the pro-apoptotic protein, BAD and decreases anti-apoptotic protein, SMN. In addition to this, we are

well on our way to producing skeletal muscle specific FKBP12 deficient mice. These will be used to test our hypothesis that increased cytoplasmic Ca^{2+} activates calcineurin, producing effects similar to those found with hind limb suspension.

In summary, we propose that skeletal muscle adaptation to microgravity represents an increase in both calcineurin and ubiquitin/proteasome activity and a shift in the balance between pro-apoptotic and anti-apoptotic pathways. We propose to test this hypothesis during the 03 year. The demonstration of the activation of these pathways by hind limb suspension will allow us then to design and test new countermeasures.

RESEARCH AREA:	**Muscle Alterations and Atrophy**
PRINCIPAL INVESTIGATOR:	**Nadia A. Rosenthal, Ph.D.**
ORGANIZATION:	**Harvard Medical School**
PROJECT TITLE:	**Molecular Mechanisms Regulating Muscle Fiber Composition Under Microgravity**
FUNDING:	**$134,384 (FY 1998); $139,162 (FY 1999); $139,162 (FY 2000)**
TOTAL FUNDING:	**$412,708**

PROJECT EXECUTIVE SUMMARY

The overall goal of this project is to reveal the molecular mechanisms underlying the selective and debilitating atrophy of specific skeletal muscle fiber types that accompanies sustained conditions of microgravity. Since little is currently known about the regulation of fiber-specific gene expression programs in mammalian muscle, elucidation of the basic mechanisms of fiber diversification is a necessary prerequisite to the generation of therapeutic strategies for attenuation of muscle atrophy on earth or in space.

Vertebrate skeletal muscle development involves the fusion of undifferentiated mononucleated myoblasts to form multinucleated myofibers, with a concomitant activation of muscle-specific genes encoding proteins that form the force-generating contractile apparatus. The regulatory circuitry controlling skeletal muscle gene expression has been well studied in a number of vertebrate animal systems. The goal of this project has been to achieve a similar level of understanding of the mechanisms underlying the further specification of muscles into different fiber types, and the role played by innervation and physical activity in the maintenance and adaptation of different fiber phenotypes into adulthood.

Our recent research on the genetic basis of fiber specificity has focused on the emergence of mature fiber types and have implicated a group of transcriptional regulatory proteins, known as E proteins, in the control of fiber specificity. The restriction of E proteins to selected muscle fiber types is an attractive hypothetical mechanism for the generation of muscle fiber-specific patterns of gene expression. To date our results support a model wherein different E proteins are selectively expressed in muscle cells to determine fiber-restricted gene expression. These studies are a first step to define the molecular mechanisms responsible for the shifts in fiber type under conditions of microgravity, and to determine the potential importance of E proteins as upstream targets for the effects of weightlessness.

In the past year we have determined that the expression of E Proteins is restricted to specific fiber types by post-transcriptional mechanisms. By far, the most prevalent mechanism of cellular control for achieving post-transcriptional regulation of gene expression is selective proteolysis through the ubiquitin -proteasome pathway. Steady-state levels of HEB message are similar in all fast and slow skeletal muscle fiber types, yet the protein is restricted to Type IIX fibers. HEB appears to be a nodal point for regulating fiber-specific transcription, as expression of the transcription factor is regulated at the post-transcriptional level. It is not clear at present whether the regulation is at the level of protein synthesis or degradation.

We are now poised to evaluate the biological role of ubiquitination in fiber specific-gene expression by controlling the post-transcriptional expression of E Proteins. The use of metabolic labelling and pharmacological inhibitors of the ubiquitin pathway will be used to identify the

mode of regulation of the Type IIX expression pattern. The potential role of specific kinases in effecting the restriction of HEB expression will be examined by using both inhibitors and activators. The results of these studies will provide the necessary information to evaluate the biological role of E proteins in controlling fiber type transitions, and in potentially attenuating the atrophic effects of microgravity conditions.

We have also recently shown that ectopic expression of the HEB protein transactivates the Type IIX-specific skeletal a-actin reporter. The 218 bp skeletal a-actin promoter drives transgene expression solely in mature Type IIX fibers. A mouse also carrying the transgene MLC1/HEB (which ectopically expresses the E Protein HEB in Type IIB fibers) forces expression of the skeletal a-actin reporter gene in Type IIB fibers.

We can now dissect the composition of this fiber-specific *cis*-element. The skeletal a-actin promoter is quite compact and has been extensively characterized in vitro for activity and binding factors. The single E box may act as a binding target of myogenic factor/HEB heterodimer to allow for IIX expression. The HEB transcription factor may recognize either the precise flanking sequences of the E Box, or perhaps interacting with other proteins bound nearby, and activating expression in Type IIX fibers. This E box will be both ablated, and alternatively, as ablation may well destroy any muscle-specific transcriptional activity, flanking sequences substituted with those surrounding the E box (E1) of the myogenin promoter. Modification of fiber-specific transgene expression will be tested in transgenic mice. The results of these studies will provide basic information on the regulatory circuitry underlying fiber specificity, and will form the basis for building appropriate transgenic regulatory cassettes to effect fiber transitions in subsequent experimental manipulations on unweighted muscles.

RESEARCH AREA:	**Muscle Alterations & Atrophy**
PRINCIPAL INVESTIGATOR:	**Dennis R. Mosier, M.D., Ph.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Motoneuron Influences on Muscle Atrophy in Simulated Microgravity Induced Muscle Atrophy**
FUNDING:	**$116,800 (FY 1998); $119,603 (FY 1999); $119,603 (FY 2000)**
TOTAL FUNDING:	**$356,006**

PROJECT EXECUTIVE SUMMARY

Many alterations in motor unit structure and function occur with exposure to microgravity during spaceflight, and could impair motor performance. While much work is ongoing to ascertain the nature of biochemical, structural, and physiological changes occurring in muscle fibers, little attention has been paid to the changes reported in motoneuron terminals at the skeletal neuromuscular junction, and in motoneuron cell bodies, during exposure to microgravity. It is unlikely that these changes, whether they occur independently or secondary to changes in the innervated muscle fibers, are without consequences for the regulation of motor unit function. Accordingly, the central hypothesis of this study is that alterations in motoneuron structure and function occur during the process of microgravity-induced muscle atrophy, and that these alterations significantly influence muscle dysfunction, adaptation, and recovery from atrophy induced by microgravity. These changes may be manifested as early structural and functional alterations in the distal motoneuron terminal, in addition to alterations in motoneuron activity produced by changes in stretch reflexes and supraspinal pathways. Initiation of alterations in motoneuron terminals may be influenced by retrograde signals from muscle which induce, as an early event, changes in intracellular calcium and transmitter release. To begin to address these hypotheses, a combination of electrophysiologic assays of transmitter release at neuro-muscular junctions, coupled with electron microscopic assays of junctional remodelling, synaptic vesicles, and intraterminal calcium, is being used to define quantitatively the nature, extent, and possible significance of changes in motoneuron terminals occurring in a mouse model of unloading-induced muscle atrophy.

During the course of this work, a technique for S-SFEMG (stimulated single-fiber electromyography) was adapted and validated for mice, allowing *in vivo* measurements of neuromuscular transmission. Data from this work suggest that: (1) unloading of skeletal muscle is associated with altered transmitter release at neuromuscular junctions, ultrastructural abnormalities, and a reduced safety factor suggesting insecure neuromuscular transmission; (2) the extent and nature of these junctional alterations vary among individual hindlimb muscles, possibly relating to differences in muscle loading and/or fiber type composition; (3) the extent of junctional alterations varies with duration of hindlimb unloading; (4) unloading of skeletal muscle may be associated with increased calcium in the motoneuron terminal, which may act as a signal inducing the observed junctional alterations; and (5) altered neuromuscular junctions in unloaded muscle retain the insensitivity to acute muscle stretch typical of normal mammalian junctions. Based on our observations, we hypothesize that junctional remodeling associated with muscle atrophy may vary over time, and may, especially in combination with other physiological stresses encountered during spaceflight (e.g., hypothermia, medication effects), pose a risk of junctional transmission failure. In the mouse hindlimb unloading model, we were unable to reproduce the full range of ultrastructural changes reported in studies of space-flown animals, and therefore suggest that additional factors (especially reloading injury and/or eccentric

contraction injury of atrophied muscle) may have contributed to this disparity. Our evidence to date suggests that transgenic over-expression of IGF-1 in skeletal muscle, which can induce junctional alterations in some systems and is proposed as a potential countermeasure for unloading-induced muscle atrophy, does not exacerbate junctional alterations in this model system. Finally, our preliminary data indicating alteration of intracellular calcium and of calcium-dependent processes within motoneuron terminals suggest the possibility of increasing calcium-binding protein expression as a potential countermeasure for the observed alterations of neuromuscular junctions with hindlimb unloading.

Our comprehensive approach using electrophysiologic and ultrastructural techniques is being extended to determine the junctional effects and tolerability of transgenic overexpression of a calcium-binding protein, parvalbumin, and to determine whether parvalbumin overexpression can ameliorate motoneuron dysfunction and/or muscle atrophy in mouse models of muscle atrophy and of neuromuscular diseases. Data obtained from this study will be useful in defining the anatomic and physiologic consequences to motoneurons of manipulations which induce muscle atrophy, and will aid in designing further experiments to determine the mechanisms influencing motor unit dysfunction occurring during space travel. Information from this study will be of value to the design and refinement of countermeasures aimed at ameliorating the deleterious effects of microgravity on human motor performance. The results of this work may also provide new insights into important clinical problems such as mechanisms influencing motoneuron dysfunction in devastating degenerative illnesses such as amyotrophic lateral sclerosis, muscle and motor nerve injury encountered in critical care settings, and the design of therapies to retard or prevent muscle atrophy produced by disuse or spinal cord injury.

NSBRI RESEARCH PROGRAM
NEUROVESTIBULAR ADAPTATION

Team Leader: Oman, C. M. MIT

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Oman, C. M.	CO-I	MIT		
Raphan, T.	CO-I	Brooklyn College		
Young, L. R.	CO-I	MIT		

NEUROVESTIBULAR ADAPTATION TEAM PROGRAM EXECUTIVE SUMMARY

NSBRI's neurovestibular adaptation research program supports research aimed at developing scientifically-based countermeasures against the vestibular problems associated with long duration space flight: space motion sickness, disorientation, oculomotor deficits, postflight postural instability and gait ataxia. Neurovestibular problems typically arise first when astronauts transition from 1-G to 0-G, unfortunately just when their physical and cognitive performance is critical for mission success and safety. Similar problems are expected on exploration class missions when astronauts make the transition from 0-G to partial G, or from 0-G to an artificial gravity environment. Our research also has potentially important terrestrial applications: An estimated two million American adults summer from chronic impairment of dizziness or difficulty with balance, and one quarter of emergency room visits include a complaint of dizziness. Balance-related falls account for more than one half of accidental deaths in the elderly.

The NSBRI neurovestibular adaptation research program addresses five major space neurovestibular risk areas, as identified by NASA Life Science's Critical Path Risk analysis project:

1. Disorientation and reduced performance on cognitive and physical tasks, including vehicle egress, especially during/after g-level changes (associated with acute spontaneous and head movement contingent vertigo, nystagmus, oscillopsia, saccadic errors, reduced dynamic visual acuity, spatial memory problems).

2. Impaired neuromuscular coordination and/or strength (gait ataxia, postural instability).

3. Impaired cognitive and/or physical performance due to spatial disorientation, motion sickness symptoms or treatments (including short term memory loss, reaction time changes, drowsiness, fatigue, torpor, irritability, ketosis) as a result of changes in g-level, or use of artificial gravity.

4. Autonomic dysfunction (including cardiovascular, respiratory, gastrointestinal, sleep and mood changes) which may be of vestibular origin.

5. Permanent impairment of orientation or balance function due to microgravity or radiation (causing chronic imbalance, gait ataxia, vertigo, chronic vestibular insufficiency, poor dynamic visual acuity)

The goals of the program are to develop countermeasures that ultimately will to allow crewmembers to:

- Avoid disorientation
- Meet physical requirements of emergencies
- Treat motion sickness without side effects
- Safely control vehicles and systems.

Nine interrelated, countermeasures-oriented research themes currently define the scope of the neurovestibular program:

1. Adaptive Generalization and Context-Specific Adaptation
2. Artificial Gravity
3. Visual (multisensory) orientation, navigation and spatial memory
4. Drug countermeasures
5. Postflight locomotion and gaze assessment
6. Neurovestibular rehabilitation
7. Vestibular effects on autonomic function
8. Effects of weightlessness, stress, isolation, immobilization, radiation and diet on vestibular function.
9. Potential mechanisms for and diagnosis of irreversible neurovestibular changes.

During the 1981-1997 Shuttle era, space neurovestibular research focused on understanding the effects of unweighting of the otoliths on the vestibulo-ocular reflex (VOR), predicting space sickness susceptibility, and measuring postflight postural stability while standing. NSBRI's current early ISS-era neurovestibular research program is investigating context specific pre-adaptation, preflight visual orientation and 3D spatial memory training countermeasures, and improving our ability to assess postflight locomotion and gaze control problems. Research is being conducted at the cognitive, behavioral, system, organ, and cellular level, using quantitative techniques in both humans and animals. The work is interdisciplinary, and involves collaborations between investigators at multiple institutions.

Our goal is to achieve risk reduction by developing scientifically based countermeasures. Basic research projects must plausibly lead in that direction. Once specific countermeasures are proposed, they will have to be proven safe, effective and practical, and their potential impact on other physiological systems understood. NASA and NSBRI define the countermeasures development process in terms of three phases, and nine levels of readiness, which range from basic research (levels 1-3) through countermeasure feasibility and development (levels 4-6) to ground evaluation and flight validation (levels 7-9). Most NSBRI research teams' activity falls in the 1-7 range.

During the Institute's first three years, neurovestibular adaptation research was conducted by a team of 21 investigators from 11 institutions, organized into three projects. An introduction to these projects is available at www.nsbri.org and also from the Neurovestibular Team web site mvl.mit.edu/Neurovestibular/Pages/home.html. Final project reports are available from NSBRI which detail the scientific accomplishments of the three projects and their implications for future research are summarized and detailed.

This report describes the development of NSBRI's neurovestibular program research strategy and the "critical path" risks which motivate it. It also provides a programmatic level review of each project's research questions, an overview of what was achieved, and how far each projects moved the neurovestibular discipline forward in terms of countermeasures readiness.

Context-Specific Adaptation of Gravity-Dependent Vestibular Reflex Responses.
(M.J. Shelhamer, Johns Hopkins University, and 5 co-investigators.)
Can vestibular reflexes be pre-adapted (pre-conditioned) in context specific ways, so astronauts can rapidly transition between Earth, weightlessness, rotating spacecraft (artificial gravity) and planetary gravity, with minimal disorientation and performance impairment ? This basic research project succeeded in obtaining fundamental evidence on several types of context-specific adaptation in both human and animal subjects. It was demonstrated that g-level and eye position

can be used as a context cue for changing the size of saccadic and smooth pursuit eye movements, and that saccadic adaptation can persist for many months. G-direction can provide a context cue for adapting the Linear Vestibulo Ocular Reflex (LVOR). In both cases, the evidence suggests that the more relevant the context cue is to the response being adapted, the more effective it is. Adaptation of the Vestibular Coriolis oculomotor response in a rotating environment – and retention of it - is of particular importance to our artificial gravity research theme. It was shown that three ten-minute periods of out-of-rotation-plane head movements produced measurable reduction of inappropriate eye movements, and adaptation was retained one week later. Some support for context specificity was found: subjects did not experience motion illusions when head movements were made in a non rotating environment. In other experiments, it was shown that the human vestibulo-collic reflex adapts much more quickly to artificial increases in head inertia after several practice sessions on consecutive days. Some progress was made using primate models to understand the underlying physiology of adaptation: LVOR adaptation was found to be specific to the frequency used. There was no correlated change in static ocular torsion resulting from head tilt. Floculectomy permanently reduced the LVOR, even after the angular VOR had recovered. Human cerebellar patients show comparable deficits. The investigators note that the "0-g flashbacks" anecdotally reported by some crewmembers are consistent with the notion of dual-state adaptation. They have also pointed out that cerebellar adaptation failure might account for a number of the oculomotor problems reported in the Russian literature after long duration spaceflight. The investigators have outlined several potential concepts for preadapting the human LVOR in astronauts. This group's participation in the JSC Neurological Function IPT has helped formulate plans for more comprehensive postflight oculomotor examinations of crewmembers by flight surgeons. In the areas where they have been working, Dr. Shelhamer's group has moved our understanding of these problems from Countermeasure Readiness level 1 to Level 2.

Visual Orientation in Unfamiliar Gravito-Inertial Environments.
(C.M. Oman, Massachusetts Institute of Technology and 4 co-investigators.)
What visual cues do astronauts rely upon to maintain their spatial orientation and sense of where they are relative to other objects and places ? Why is living in a three dimensional structure like the MIR space station disorienting ? What visual cues normally provide us with cues to the gravitational vertical on Earth ? Does inadvertent use of these same cues cause disorientation when astronauts live in weightlessness ? Does 1-G training in simulated 0-G real or virtual environments improve spatial orientation, spatial memory and task performance ? Using tumbling room and mirror bed devices, the group showed that compelling visual scenes can produce much larger illusions of subjective tilt than had previously been thought. For example, many supine subjects feel erect which viewing an environment which is visually upright with respect to their bodies, resulting in an interesting "levitation" sensation if subjects elevate their arms. The strength of tilt illusions also depends on the gravitational "polarity" of objects in the scene. This attribute depends both an object's usual orientation in 1-G everyday life, and on its means of apparent physical support. A correlation between tilt illusion susceptibility and age was found. Results on the polarity of objects in a visual scene can be used to update and extend NASA human factors standards on spacecraft visual verticals, and also suggest specific countermeasures. For example, placing pictures of "gravitationally polarized" objects on walls could make older people less prone to falls, and astronauts less prone to visual reorientation illusions. In a collaborative project with the Cardiovascular team, the investigators found that if a scene is sufficiently polarized and realistic, e.g. produced using an inclined mirror, the resulting tilt illusion can produce transient cardiorespiratory changes, further evidence of visual/vestibular-autonomic coupling. The team has also studied 3D spatial memory in a task analogous to that confronting astronauts in the node module of a space station. Though the experiments were

performed in 1-G, spatial memory and learning was not strongly dependent on the gravitational orientation of the subject, nor on whether a virtual or real training environment was used. Instead, performance depended on the mental image and mnemonic strategies used, and correlated with performance on conventional 2 and 3 dimensional mental rotation ability, and visual field dependence. Training the subjects on generic 3D orientation strategies was found to help. Skills acquired were shown to transfer to a second environment, and were retained for at least several weeks. The training method could be used as the basis of a generic preflight spatial orientation training procedure for astronauts. Lastly, this group has studied the limbic coding of 3D orientation in 1-G and in an animal model in parabolic flight. Head direction cells in the anterior thalamic nuclei of rats retained normal visual environment-referenced responses in zero g and hypergravity when on the floor or walls. When crawling on the ceiling, directional specificity was frequently lost. However, when the animal was on the ceiling in 0-G, reversals in cell preferred response direction were found. The investigators believe this phenomenon is the neural correlate of visual reorientation illusions described by humans in analogous situations. The finding helps explain rat place cell results obtained on the Neurolab Spacelab mission. The team has succeeded in defining a scientific basis for preflight visual orientation training, the main goal of the project. In terms of countermeasures readiness, Dr. Oman's team has moved several concepts from Level 2 through Level 5.

Advanced Techniques for Assessment of Postural and Locomotor Ataxia, Spatial Orientation, and Gaze Stability.
(C. Wall, Massachusetts Eye and Ear Infirmary and 6 co-investigators.)
Astronauts returning from long duration missions typically report postflight problems with standing, walking, and gaze stabilization. The longer the exposure to weightlessness, the more profound and long lasting the postflight deficits usually are. This group has developed a number of quantitative methods for measuring gaze, head and body stability during normal and perturbed locomotion. One technique, "Ideal Trajectory Analysis" (ITA) yields a measure of kinematic deviation from an ideal sinusoidal body center of mass trajectory. Analyzing data from subjects performing a repeated "stepping up" task, the investigators can statistically discriminate normals and patients. ITA has been used to evaluate the effects of vestibular rehabilitation in patients, and may also prove useful for assessing the performance and rehabilitation of returning astronauts. The project has also better defined the relationship between head motion and walking speed, and between eye motion and visual target distance. During normal walking, the head pitches in phase with gait so it aimed consistently at a point called the "Head Fixation Distance", located about one meter ahead, and independent of gaze fixation distance. This is partly the result of a linear vestibulo-collic reflex (lVCR), responding to the vertical kinematic component of walking. When looking at a point far away during normal walking, the angular vestibulo-ocular reflex provides appropriate ocular stabilization. Looking near invokes the vertical LVOR, which may be affected by spaceflight. The angular vestibulo-collic reflex is dominant at low walking speeds and when viewing objects far away, and the lVCR is dominant at the higher optimal walking speeds, and when viewing near targets. Astronauts often report postflight difficulties while walking around corners, so the group has studied the eye, head and body response in normal subjects while walking straight as compared with walking a curved path, and showed how responses align with the resultant gravito-inertial acceleration. The group has also measured responses to perturbed gait by measuring the response of different body segments (head, trunk, legs) to a controlled mechanical perturbation of the foot after heel strike. Vestibular patients require several more steps to recover than normals. Several of these test techniques are now well defined, and are candidates for evaluation as postflight neurovestibular assessment tools to assess the return of normal function in astronauts. Dr. Wall's team has moved these from Countermeasures Readiness Level 1-2 to Readiness Level 5.

Looking to the future, several of these promising countermeasures concepts are near the point where they can be transitioned to JSC for further evaluation and development. In the important area of context-specific preadaptation training development, much work remains to be done. We hope to be able to expand the program to include several new areas of emphasis, including artificial gravity, postflight neurovestibular rehabilitation, and improved anti-motion sickness drugs.

RESEARCH AREA:	**Neurovestibular Adaptation**
PRINCIPAL INVESTIGATOR:	**Mark J. Shelhamer, Sc.D.**
ORGANIZATION:	**Johns Hopkins School of Medicine**
PROJECT TITLE:	**Context-Specific Adaptation of Gravity-Dependent Vestibular Reflex Responses**
FUNDING:	**$304,496 (FY 1998); $300,549 (FY 1999); $476,275 (FY 2000)**
TOTAL FUNDING:	**$1,081,320**

PROJECT EXECUTIVE SUMMARY

When we move about in the environment, we constantly make use of reflexive motor adjustments in order to maintain posture and balance in reaction to disturbances. Two such motor activities are the movements of the head and the eyes. Impairment of these motor reflexes can lead to disorientation and reduced performance in sensorimotor tasks (such as piloting of spacecraft). Therefore, the adaptive abilities of these systems are important to prevent mishaps during changes in environmental conditions (e.g. gravito-inertial force, gif).

In the absence of a normal earth gravity field, the dynamics of head stabilization, and the interpretation of vestibular signals that sense gravity and linear acceleration, are subject to change. Transitions between different gif environments – as during different phases of space flight – provide an extreme test of the adaptive mechanisms that maintain these reflexes. During extended space flight, crew members may live in artificial gravity and make transitions to weightlessness, planetary exploration, and return to Earth. If they learn sensorimotor skills such as piloting in the normal gravity of Earth, will they be able to perform them adequately in the weightless or the artificial gravity environment? More generally, can people have two different sets of vestibular reflexes, which they are able to switch between rapidly? Are there procedures that could help to transfer (or to inhibit) training from one situation to another? These are the main types of questions addressed by our work. The overall goal of this project is the study of context-specific vestibular and oculomotor reflexes. Special emphasis is placed on the use of gif as a context cue for switching between adapted reflexes. The general approach is to adapt a specific motor response (saccades, VOR, VCR) in one way (e.g. increase in gain) in one gif condition, and another way (e.g. decrease in gain) in another gif condition, and then see if the gif condition itself (the context cue) can recall the previously learned adapted responses.

The knowledge gained from our studies will help us to design adaptation strategies (pre-flight and in-flight) to assist flight crews in making transitions between different gravitoinertial force situations, and can provide design data for spacecraft facilities (artificial gravity, exercise centrifuge) by delineating the limits of human adaptive capabilities.

A prerequisite to the use of context-specific adaptation procedures as a countermeasure is to identify those responses that need to change in a context-specific manner during space flight. There are many physiological changes that occur during flight, but not all of them are adaptive in the sense of bringing performance back to the normal pre-flight level. One must also think in terms of possible detrimental effects during long-duration flight. If reflexes become inappropriately calibrated during extended flight, then this "incorrect" calibration may generalize to the planetary gravity phase. There is certainly evidence that this occurs, as evidenced by difficulties with posture and locomotion immediately after shuttle flight.

Some reflex responses that develop in flight are inappropriate for planetary gravitational fields, while perfectly acceptable for 0g. An example is the putative reinterpretation, in space, of all otolith stimulation as translation (rather than tilt). As there is no true tilt (change in orientation with respect to gravito-inertial vector) in space, the absence of tilt responses is acceptable there. However, when this response configuration generalizes to a planetary gravity environment (whether Earth [1 g] or Mars [0.38 g]), it is inappropriate. Thus we might tailor context-specific adaptation to maintain acceptable planetary responses while in flight, in association with artificial gravity or another stimulus arrangement that simulates one requiring tilt responses.

As an example, two responses which may be amenable to context-specific adaptation follow:

- If artificial gravity is used during long-term flight, it may be desirable to maintain the appropriate sensorimotor calibrations for the rotating (artificial gravity) and non-rotating (weightless or planetary) environments. Normally, exposure to a rotating environment induces disorientation and inappropriate reflex components, due to the action of cross-coupled (Coriolis) rotational stimuli on the vestibular system. Adaptation would involve training subjects to have the appropriate reflex calibrations while rotating and not rotating, and to switch between the two sets immediately when switching environments.
- Adaptation to the space environment may involve reinterpreting otolith (linear acceleration) stimulation as arising from translation, rather than from combinations of translations and tilts with respect to gravity as on earth (otolith tilt-translation reinterpretation: OTTR). One pre-flight adaptation strategy would be to reduce the tilt component of motor responses to mimic the flight environment (while alternately presenting stimuli to retain the tilt component, to enhance the eventual return to a gravity environment).

Related issues addressed in our experiments include determining 1) effective adaptation schedules, 2) if gravity can be an effective context cue and for which responses, and 3) the role of the cerebellum. The role of the cerebellum in these adaptations is important as a point of fundamental knowledge, but it has serious practical import as well. If, as seems likely, the cerebellum is adversely affected by space flight, then its ability to implement adaptation-based countermeasures is suspect. We would be remiss to propose countermeasures based on adaptation of vestibular responses without assessing the involvement of the cerebellum.

Outline of Individual Sub-Projects

Various experiments investigate the behavioral properties, neurophysiological bases, and anatomical substrate of context-specific learning mechanisms. We use otolith (gravity) signals as the contextual cue for switching between adapted states of the saccadic system, the angular and linear vestibulo-ocular reflexes, and the VCR. (By LVOR we mean the oculomotor response – horizontal, vertical, and torsional – to linear translation of the head and body.)

Context-specific saccade adaptation. We have evidence for context-specificity in human saccades. Two sets of parabolic flight experiments examined the use of instantaneous gravity level (alternating 0 g and 1.8 g) as a context cue for adapted saccadic eye movements. Saccades (rapid eye motions that move the eyes between targets) can be adaptively altered by presenting a target, then moving that target to a new location before the eyes can get to its first location. After several trials, an adaptive sensorimotor mapping takes place, so that the eyes move directly to the new target location when presented with the original target. Ground experiments at Johns Hopkins successfully used vertical eye position, horizontal eye position, head roll tilt, and upright/supine posture as context cues, so that saccades are increased in size in one context (when subjects look upward, or tilt their heads to the right, or are seated upright), and decreased

in size in the other context (when subjects look down, or tilt their heads to the left, or are supine). Data from parabolic flight indicate that g-level also can serve as an effective context cue.

Context-specific LVOR adaptation. We demonstrated the ability to use a gravity cue (head orientation) as a context for switching between two different adapted versions of the linear VOR. The gain of the LVOR can be adaptively changed by having the subject view a visual field that moves with him or her on the sled (driving the gain down, since no eye movements in response to head/body translation are required to stabilize the visual field) or view a visual field that moves opposite to sled motion (driving the gain up). We have been able to induce changes in gain that are associated with head roll tilts (context cues) in different directions.

Properties of AVOR and LVOR in squirrel monkey. In the squirrel monkey, we completed baseline investigations of the dynamics of the AVOR with high frequencies and accelerations, revealing interesting nonlinearities which must be understood before adaptive effects can be investigated. Monkey LVOR adaptation studies were also performed, demonstrating adaptive increases and decreases. Torsional eye movement responses to the linear translations did not change significantly after adaptation, suggesting that the translational and tilt components of the LVOR (horizontal and torsional eye movements, respectively) may not be closely coupled. This has implications for paradigms designed to adaptively change tilt-translation interpretation.

Pursuit and the LVOR in humans and in rhesus money. Results in animals indicate that the LVOR is abolished after flocculectomy, and it is greatly impaired in humans with vestibular deficits as well. Pursuit deficits mirror these changes in the LVOR. This suggests that pursuit and the translational LVOR are tightly linked. A separate set of experiments has demonstrated context-specific adaptation of pursuit gain in humans and monkeys. These two results together may form the basis for a powerful strategy to adapt the otolith-mediated translational LVOR.

Properties and adaptation of head-neck reflexes. Experiments at Baylor College of Medicine on adaptation of the VCR also show evidence of context-specificity. These experiments have established baseline properties of the response along different axes, in terms of mathematical models. Adaptation to an artificial increase in inertia of the head has been demonstrated, as manifest by a decrease in head oscillation during body perturbations. The appropriate adapted response was stored by the head-neck control system even after subsequent re-adaptation back to normal inertia: the system responded appropriately to each inertial load to keep head oscillations at the same level. This capacity to switch between two sets of system parameters persists for at least 35 days after the initial adaptation: the appropriate head damping occurred immediately for both normal and increased inertia loads, showing that two sets of damping parameters can exist simultaneously and be switched in and out as needed.

Adaptation to a rotating environment. Short-radius centrifugation (a form of artificial gravity) is a promising potential countermeasure to long-term weightlessness. Unfortunately, it has a number of side effects related to the unexpected effects of head movements in the rotating environment. Transitions between the artificial gravity (rotating) and weightless (non-rotating) environments will likely cause additional problems. Experiments at MIT are investigating the extent to which these side-effects can be overcome through adaptation. Head movements during centrifugation induce discomfort, non-compensatory vestibulo-ocular reflexes, and illusions of body tilt. Significant adaptation occurred following a series of experimental sessions of head turns during rotation in the light, such that these detrimental effects were reduced.

Key Findings and their Implications

- Saccadic eye movements can be adapted in a context-specific manner, using a number of different context cues. The more relevant the context cue is to the response being adapted, the more effective it seems to be in context-switching (e.g. horizontal eye position is a more effective cue for horizontal saccade adaptation than is vertical eye position).
- The magnitude of gif (during parabolic flight) can be used as a context cue for switching between adapted saccade states. There is evidence for retention of this adaptation after 8 months. The lunar and Martian g levels can recall adaptations imposed during 0 g. (This and the above result satisfy a modified version of aim 1 of the original proposal; the original aim involved adaptation of the AVOR, but saccade adaptation is more easily accomplished in parabolic flight, and there is evidence that saccade accuracy may be adversely affected during flight, due to alterations in static torsional eye position. The essential component of the aim – use of g level as a context cue – was achieved.)
- Compensatory eye movements made in response to translational (LVOR) can also be made context-specific, using the orientation of gravity with respect to the head (head tilt) as a context cue. For inter-aural translations, head roll is a more effective context cue than is head pitch. This is analogous to the situation with saccade adaptation: the closer the context cue is to the response being adapted, the more effective it is. (This satisfies a modified aim 2 of the proposal. It was proposed to adapt phase rather than gain, but gain adaptation has turned out to be easily accomplished, and has more countermeasure relevance.)
- Sensorimotor adaptation to head movements during short-radius centrifugation (23 rpm, 1 g at the feet) occurs, as quantified by measures of inappropriate vertical eye movements, motion sickness, and illusory tilt. Three ten-minute adaptation sessions produced adaptation that was retained (at reduced level) a week later. Adaptation to head movements to one side did not generalize to head movements in other directions. Full adaptation did not take place; while motion sickness disappears after 10 adaptation sessions, vertical nystagmus and illusory tilt do not. Context-specificity of the adaptation is apparent since subjects did not experience motion illusions when off the centrifuge between test sessions. (This satisfies aim 3 of the original proposal. Subjects can acquire adaptation to short-radius centrifugation, and move between rotating and normal environments without detriment.)
- Properties of the head-neck control system (VCR) in three dimensions (roll, pitch, yaw) can be adequately modeled by a relatively simple, 2^{nd}-order linear system, plus a single dead-zone nonlinearity. Adaptation of this system to changes in head inertia can be induced. This adaptation can be made dual-state, such that the appropriate neural control mechanisms for head stabilization change modes immediately upon a change in head inertia. (This satisfies aims 4 and 5: modeling of the vestibular contribution to head stabilization has been accomplished, short-term adaptation has been demonstrated, and some measure of context-specific adaptation to immediate and repeated changes in head inertia has been shown.)
- Bilateral removal of the flocculus and paraflocculus in rhesus monkey produced almost complete loss of the horizontal LVOR (even after the angular VOR had recovered). Likewise, human cerebellar patients have comparable defects in pursuit and the LVOR, while the AVOR appears to be controlled independently. This suggests that the vestibulocerebellum plays a critical role in the generation of the LVOR, and that there is a tight relationship between the generation of the LVOR and smooth pursuit. This has implications for countermeasures that are based on adapting translation versus tilt responses mediated by the otoliths. (This satisfies multiple aspects of aim 6: the role of the vestibulocerebellum in the LVOR, and the role of pursuit in the generation of the LVOR.)

- A separate experiment showed systematic variations in the axis of eye rotation at different vertical elevations, during pursuit, AVOR, and LVOR. Axis tilts for pursuit and LVOR were almost identical, and different from that for the AVOR, again showing a close relationship between neural processing for pursuit and the LVOR. (This satisfies the remaining portion of aim 6: assessment of axis of rotation in pursuit, LVOR, and AVOR.)
- Context-specific adaptation of smooth pursuit eye movements has been demonstrated in both humans and rhesus monkeys. Using vertical eye position as a context cue, the initial acceleration of the eyes, when presented with a moving target, can be made to decrease with the eyes elevated, and to increase with the eyes depressed. This has implications for context-specific adaptation of some types of otolith-mediated responses, which seem to be at least partly expressed through the pursuit system (see above). (This partially addresses aim 8, which was intended to determine the role of the vestibulocerebellum in context-specific LVOR adaptation. Although the original aim was not dealt with directly, progress was made in the general area by determining the role of the cerebellum in pursuit and the LVOR, and by demonstrating context-specific adaptation of pursuit.)
- LVOR gain adaptation was induced in squirrel monkeys, and was specific to the frequency used for adaptation. Following adaptation of LVOR gain, there was no significant change in the torsional eye movements to head tilt, suggesting that the responses to head tilt and head translation are not tightly coupled. (This is the initial stage of aim 7, meant to determine the role of the vestibulocerebellum in the adaptive control of the gain and phase of the LVOR. Other parts of aim 7 are still under investigation, and have had to await the development of equipment and procedures for LVOR adaptation in the monkey, and lesioning of same.)

As pointed out above, although much of aim 8 (role of the vestibulocerebellum in context-specific LVOR adaptation) was not addressed directly, a number of related experiments in humans (not all of which were originally proposed) have made a significant contribution to the overall goal of this aim. In particular, elucidation of the role of the cerebellum in pursuit and the LVOR, demonstration of the close connection between these two responses, and the production of context-specific adaptation of both the LVOR and pursuit, all contribute to understanding the role of the cerebellum in these adaptive processes. This made some of the specific proposed monkey experiments relatively less important. The animal work continues to have relevance, however, in that it will allow more extensive testing over a range of stimulus parameters, and localization of those cerebellar pathways which contribute to adaptation.

Additional Implications, Relationship to NASA Critical Path Issues

Neurovestibular problems have been identified and listed on the NASA "Critical Path Roadmap" for serious problems that could affect a mission to Mars. Some indication of the range of problems and their severity is found in the May 1997 "Final Report of the NASA Task Force on Countermeasures," which states: "Based on the experience of both the cosmonauts and the astronauts, it is apparent that the ability to egress suddenly will be limited unless effective countermeasures for the loss of neuromuscular performance are identified and adhered to rigidly during prolonged spaceflights" (p. 9). Specific problems listed in the report include changes in eye-head coordination, decrements in postural control, sensory illusions such as otolith tilt-translation reinterpretation, and "flashbacks" between 1g and 0g states with associated motor dysfunction. Concerns were raised for the effects of these problems on vehicle control and unassisted egress. The issue of "flashbacks" is especially interesting relative to our work, as it indicates the simultaneous existence of two adapted states (one for 0g and another for 1g). Knowledge about how to avoid such inadvertent flashbacks, as well as how to make use of contextually-gated dual-state adaptation, is the central aim of all of our studies in this project.

An especially useful aspect of our parabolic flight experiments is that we fly in consecutive years. With the same subject tested each year, we can assess how much the 0 g responses have been maintained throughout the intervening period of 1g exposure. This is particularly germane to Mars missions, when gravity-based responses which may have been trained before flight may have to be recalled in the Martian gravity environment many months later.

Not only do our studies provide valuable information for the development of countermeasures, they will also provide basic information on adaptive neurovestibular processes. This is especially true of experiments dealing with the role of the cerebellum in motor control, and signal processing of otolith information for the generation of reflex responses in different environments.

One specific clinical implication of these studies is in the area of vestibular rehabilitation (and physical rehabilitation in general). Rehabilitation exercises are generally learned and carried out under supervision in a clinical setting. There is the possibility that inadvertent contextual cues in this setting will be associated with improved performance while in the clinical setting, which will not transfer completely to settings of normal daily living. In this respect, it is useful to know what context cues are most effective, what types of responses can be made context-specific, and how to avoid such context-specificity when it is detrimental (i.e. when generalization is desired).

RESEARCH AREA:	**Neurovestibular Adaptation**
PRINCIPAL INVESTIGATOR:	**Charles M. Oman Ph.D.**
ORGANIZATION:	**Massachusetts Institute of Technology**
PROJECT TITLE:	**Visual Orientation in Unfamiliar Gravito-Inertial Environments**
FUNDING:	**$324,700 (FY 1998); $338,693 (FY 1999); $338,688 (FY 2000)**
TOTAL FUNDING:	**$1,002,081**

PROJECT EXECUTIVE SUMMARY

NASA Life Science's "Critical Path Roadmap" report recognized that the most overt change affecting an astronaut in space flight is the immediate response of the neurovestibular system to changes in gravity level. The large majority of astronauts experience some degree of space motion sickness, inflight disorientation, as well as postlanding sickness, vertigo, and ataxia. Postlanding effects are more severe after long duration missions, which suggests that neurovestibular adaptation to weightlessness – originally thought to be complete after 3-5 days – is in fact a longer process, occurring over a timescale of weeks to months. At first, the neurovestibular problems on the early Mercury, Gemini, and Apollo flights were attributed only to the effects of weightlessness on the inner ear vestibular organs. However, crewmembers in the larger Skylab, Spacelab, and MIR vehicles have described disorientation episodes, visual reorientation illusions, spatial memory and navigation difficulties, and EVA acrophobia in which vision clearly plays a major etiologic role. NASA's Critical Path Roadmap defines spatial disorientation and reduced performance on associated cognitive and physical tasks as one of the primary biomedical risks of spaceflight.

How do we know our location, orientation, and motion of our body with respect to the external environment ? On earth, gravity provides a convenient "down" cue. Large body rotations normally occur only in a horizontal plane. In space, the gravitational down cue is absent. When astronauts roll or pitch upside down, they must recognize where things are around them by a process of mental rotation which involves three dimensions, rather than just one. While working in unfamiliar situations they occasionally misinterpret visual cues and experience striking "visual reorientation illusions", in which the walls, ceiling, and floors of the spacecraft exchange subjective identities. VRIs cause disorientation, reaching errors, trigger attacks of space motion sickness, and potentially complicate emergency escape. MIR crewmembers report that 3D relationships between modules - particularly those with different visual verticals - are difficult to visualize, and so navigating through the node that connects them is not instinctive. Crew members learn routes, but their apparent lack of survey knowledge is a concern should fire, power loss, or depressurization limit visibility. Anecdotally, experience in mockups, parabolic flight, neutral buoyancy and virtual reality (VR) simulators helps. Unfortunately, our understanding of how our sense of place and orientation is coded in three dimensions in the brain is incomplete.

The role of the NSBRI neurovestibular adaptation team is to do the critical experiments that provide a rationale and methodology for scientifically based countermeasures against inflight and postflight disorientation and motion sickness. The research spans Countermeasures Readiness levels 1-6. Our specific project focuses on the role of visual cues in disorientation. Countermeasures under consideration or active development potentially generic and mission specific preflight visual orientation training using virtual reality and other simulation techniques. Also human factors standards for use of visual polarity and architectural symmetry cues, and the

design of escape path signs, allocentric visual landmark systems, and you-are-here maps. Some of these are mature enough so that during the next year, we plan to begin working with the NASA JSC Countermeasures Evaluation and Validation Project to initiate non-advocate, evidence based review and formal validation and implementation of some of our concepts. Meanwhile, a member of our research team (J. Richards) is spending 3 months at JSC this fall, working with Dr. Jon Clark, a flight surgeon who leads the JSC Neurological Function Integrated Project Team, to compile a more detailed and quantitative record concerning the actual operational incidence of visual orientation problems in the NASA-MIR and ISS programs.

The three major research themes (specific aims) of our project and the principal findings are:

1) Human visual orientation. (I. Howard, et al, York University). We have studied how visual cues determine spatial orientation and how ambiguous cues cause visual reorientation illusions. Using an 8 foot "tumbling room" at York University, whose interior was furnished with tables, chairs, bookshelves, etc. we investigated the conditions in which the perception of self orientation with respect to the vertical is dominated by gravity, the visual frame of reference provided by the room's realistic interior, or by the principal axis of the subject's body. There is a natural tendency to perceive the feet as "down". It has long been known that moving visual scenes can produce compelling illusions of self motion (e.g. flight simulators and wide screen movie theaters), but it was not understood that motionless visual scenes could produce very large sensations of static tilt under certain circumstances. Howard and Hu (2000) showed that when gravitationally supine subjects viewed the interior of the furnished room that was similarly tilted 90 degrees with respect to gravity, so that it appeared upright with respect to their body, a majority of subjects reported they felt gravitationally upright. We call this a "Levitation Illusion". If subjects extended their limbs above their supine body, their limbs felt weightless. The strength of the illusion has been systematically studied in a large group of subjects with the room and the subject in all the different possible orientations, modulo 90 degrees. In certain other relative orientations, subjects experienced visual reorientation illusions – for example they perceived the floor of the room as a ceiling. We were surprised to see that susceptibility to the levitation illusion consistently increased with the age of the subject. Vestibular function is known to degrade with age, and the association between the orientation of familiar visual objects and gravity (which we refer to as "visual polarity") is probably a learned phenomenon. In a related experiment, we also constructed a novel "mirror bed" device, which allowed us to quantify how the object property we refer to as "visual polarity" determines the strength of a VRI. A subject lying gravitationally supine in the bed views the laboratory through a mirror mounted at 45 degrees over his head. When strongly polarized objects are in view, the subject interprets the view as horizontal, and feels subjectively almost upright. The sensation of tilt is sufficiently compelling to produce visual-autonomic responses. When weakly polarized objects are seen, the subject feels nearly supine. Intermediate tilt perceptions can be created by manipulating the polarity (type and arrangement) of objects in the visual scene. Some objects (e.g. desks, chairs, saucers) have "intrinsic" polarity, because in daily life they are consistently seen in a specific orientation with respect to gravity. Other objects (e.g. blocks, pens, books) seem to have no intrinsic polarity until they are placed "on" another object. In this case, through their physical relationship the pair acquire what we refer to as "extrinsic" polarity. Understanding how the relative orientation of gravity, body axis and the visual scene interact is potentially very important for astronaut training, and also in entertainment and clinical applications. Strongly polarized objects and pictures may prove useful in reducing the incidence of disorienting VRIs in space station modules. Placing strongly polarized pictures in staircases might help some elderly people be less prone to falling.

2) Three dimensional spatial memory and learning. (C. Oman, et al, MIT and W. Shebilske, Wright State Univ.) On Earth, humans have a remarkable ability to keep track of their orientation and position relative to local landmarks. However, most large body rotations are made about the body axis which is aligned with the gravitational vertical. What are the limits of human ability to imagine, orient and navigate in a weightless environment, where one is free to turn completely upside down ? MIR astronauts reported great difficulty visualizing the relative orientation of different modules on the station, and remaining oriented when traversing the central node module. Similar problems are anticipated on the ISS. Can spatial abilities in such 3D environments be improved by preflight training ? Most navigation and spatial memory research has addressed only the terrestrial situation. To find out, we have conducted a series of four experiments on human spatial memory. We designed a 3D spatial task analogous to that confronting astronauts trying to learn the spatial relationships between the six entrance hatches in a space station node module of a space station. Subjects were placed in the center of a small six sided room. A picture of an easily recognized and remembered object was located on the center of each wall. Subjects had to memorize the relationship between the pictures, such that they could predict which picture would be where, even after the room was rotated about them into any one of 24 possible relative orientations. Subjects would be told their orientation relative to one of the walls, or shown the pictures on two of the walls, and then in darkness indicate the relative direction to a specific unseen picture. All six pictures would appear, and the subjects would have a few moments to study the relationships between the pictures before the next trial began. Some constraints were put on relative room orientation during the first two dozen trials to facilitate initial learning. However, by the time the subjects had completed sixty trials, most were able to reliably predict the direction of a specific target picture from any arbitrary orientation. The experiment was conducted using a head mounted display system to render a virtual room. However, it was repeated using a physical room, and very similar results were obtained. Tests also showed the gravitational orientation of the subject had little effect on the subject's ability to perform the task. However, the ability to do two and three dimensional mental figure rotations and recognize imbedded figures, as measured using conventional paper-and-pencil forms similar to those found on IQ tests, did consistently correlate with performance in each of our studies where we have tested it. Exit questionaires suggested our subjects chose to remember the relationships amongst the figures as they would appear with the room in a specific "baseline" orientation – often the orientation they encountered it at the beginning of the test. Many discovered they could memorize opposite pairs of objects, and learn the relationships between groups of three objects, from which the relative direction of all six could be inferred. Prior explanation and practice with the baseline orientation, pairs, and triads concepts tended to improve performance as compared to a control group, but we believe many of the control group subjects discovered these or similar techniques on their own. The best performers were the subjects with strong 2&3D mental rotation scores. Many of this group said they were able to visualize the room interior, and mentally rotate themselves or the room, and make the correct judgement, only occasionally falling back on mnemonic rules like pairs and triads. To see whether subjects "learned how to learn" the task, in some of the experiments we trained subjects in two successive environments. As we hoped, they usually learned significantly faster in the second. In one experiment we brought subjects back in for retesting and found ability was retained one day, one week, and even one month after initial training. Another experiment showed that learning with randomly chosen rather than grouped (blocked) sets of room orientations enhanced ultimate performance. We are currently working on extending the paradigm to measure spatial memory across two previously learned modules, one of which is unseen. We want to know if coalignment of the

baseline memorized module orientations is critical for performance. MIR crewmembers reported great difficulty visualizing the relative orientation of adjacent modules when the baseline orientations (established by equipment arrangements and visual verticals) was not coaligned. Our ultimate objective is to develop a methodology/pedagogy for generic and mission specific ISS preflight visual orientation training. Another application of this paradigm is in the design and evaluation of emergency escape route markings and systems of visual landmarks within modules that help crewmembers keep track of the principal axes of the ISS.

3) Neural coding of spatial orientation in an animal model. (J. Taube, et al, Dartmouth) Using a rat animal model, we have conducted experiments in our ground laboratories and in parabolic flight to better understand how our sense of place and direction is coded in 3 dimensions. In rats and primates, "head direction" cells have been found in the limbic system that appear to code head direction in a gravitational horizontal plane, independent of the animal's location, and roll or pitch of the head up to 90 degrees. The direction of maximum response ("preferred direction") lies in a fixed direction which varies from cell to cell. Under 1-G conditions, moving a prominent visual landmark around the animal results in a corresponding re-orientation of the preferred directions of all HD cells by the corresponding angle, a phenomenon that corresponds to the familiar human experience of emerging onto the street level from a subway, and reorienting your sense of direction based on viewing a familiar landmark. Until this project began, the response of HD cells had been studied only in a gravitationally horzontal plane. We have conducted a series of 1-g laboratory experiments in which rats are trained to crawl up a wall, across a ceiling (hanging upside down) and down the opposite wall, in an apparatus that allows us to verify the 3D response characteristics of HD cells, and infer whether the response sensitivity remains anchored by gravity to a horizontal plane, or whether the response coordinate frame of the cell re-orients to the plane on which the animal is locomoting. Results of these 1-g experiments indicate that cells in some animals continue to show robust direction specific firing in the same world-centered reference frame when the animal is walking upside down, and response on the walls depends on the wall and whether the animal was going up or coming down, as expected. In other animals, the cells lose their direction specific firing on the ceiling. We suspect that some animals may be better at remaining oriented on the ceiling than others. In a separate series of experiments, we have also studied HD cell response chararacteristics in parabolic flight in a test chamber that was visually symmetrical in an up-down direction. All cells HD cells studied maintained their direction specific discharge when the animal was on the floor or the wall of the chamber. However, when placed on the ceiling of the chamber, HD cell directional specificity was frequently lost. However, in some cases, the preferred direction of HD cell response reversed across the visual axis of symmetry of the cage, as it would be expected to do if the cell's response coordinate frame reoriented to the ceiling. When humans roll inverted in parabolic flight and put their feet on the ceiling of the aircraft, they experience a visual reorientation illusion in which the ceiling seems like a "floor", and the left-right axis is reversed. We believe this is the first demonstration of the limbic correlate of a human 0-G spatial orientation illusion. Corroborating evidence has also recently come from space shuttle Neurolab experiments on limbic "place" cells which we believe are driven by HD cells (Knerim, et al 2000). Our results have suggested several important physiological questions, such as what mechanisms cause HD cells to lose their directional sensitivity ? What kind of disoriented behaviors does it produce ? Our experiments provide important insights on the role played by gravireceptors in stabilizing the human sense of place and direction not only in astronauts, but also in vestibular and Alzheimer's disease patients. Funding from NASA has allowed us to pursue the anchoring role of gravity as a major theme,

and provided access to the unique facilities required for parabolic flight experiments. Involvement in the design of the experiments has helped all members of our project team form appreciate the close relationships between cognitive and cellular events.

It is never possible to predict the direction that an aggressive research program will take, but in retrospect we met or significantly surpassed our original goals in most areas, as defined in our 1997 proposal. Our discovery of 90 degree static 1-G visual reorientation illusions and their strong age dependency, the development of the mirror bed VRI technique, and its use as a research tool to quantify the strength of visual polarity cues and for vestibular autonomic research all were not anticipated. At the suggestion of advisory committees, our work on orientation and navigation has focussed on development and validation of our "virtual node" experimental paradigm, and comparison with a physical node. The physical node work was not originally proposed. However this focus has now led us to specific concepts for generic 3D spatial memory training, and positioned us well for further research on 3D navigation, and for evaluation of specific signs and landmarks. We have demonstrated a strong correlation between performance in our 3D spatial memory task and measures of mental rotation ability, and not just field dependence, as we originally proposed. Our study of the effects of foot pressure cues is still in progress. We have not attempted to assess the effects of an individual's "gravireceptor bias", as originally proposed, nor have we studied postural responses in response to scene movements. The latter study was to be part of a collaboration with another project which was not funded. Our development of an animal model for visual reorientation illusions in 1-g and 0-g has ultimately been successful, although it has proven more difficult than first anticipated to train the animals to crawl upside down across a gridded ceiling, so some of this work is still in progress. Another measure of our success is that there have been significant collaborations between investigators at the principal research sites and investigators on other teams. Dr. Oman participated in the design and performance of Dr. Taube's experiements; Dr. Shebilske designed the original "node" paradigm used by the MIT investigators. Dr. Howard and Dr. Oman have collaborated on the design of the VRI experiments, and Dr. Howard's mirror bed technique has been employed by Drs. Ramsdell and Wood of the cardiovascular team in their study of transient cardio-respiratory responses to visually induced tilt.

RESEARCH AREA:	Neurovestibular Adaptation
PRINCIPAL INVESTIGATOR:	Conrad Wall, III, Ph.D.
ORGANIZATION:	Harvard Medical School
PROJECT TITLE:	Advanced Techniques for Assessment of Postural and Locomotor Ataxia, Spatial Orientation, and Gaze Stability
FUNDING:	$340,790 (FY 1998); $347,988 (FY 1999); $305,719 (FY 2000)
TOTAL FUNDING:	$994,497

PROJECT EXECUTIVE SUMMARY

Adapting to microgravity is not the only balance difficulty astronauts face. Major postflight problems include difficulties with standing, walking, turning corners, climbing stairs and other activities that require stability of upright posture and gaze. These difficulties inhibit astronauts' ability to stand up, bail out, or escape from the vehicle during emergencies and to function effectively when leaving the space/shuttlecraft after flight. Thus it is important to understand the cause of these profound impairments of posture, gaze and locomotion stability in many returning astronauts (and in vestibular patients), and determine how they can be quantified.

Any developed countermeasure must be tested to determine its effect on gait stability, particularly under those conditions that are most troublesome following spaceflight. These countermeasures must be tested with valid and reliable tools.

This project's aims were to develop quantitative, parametric approaches for assessing gaze stability and spatial orientation during normal gait and when gait is perturbed. It has produced two new findings that are key to the understanding of human locomotion and a novel way of characterizing locomotor disturbances that are described below.

1. Understanding movements of the eyes, head, and body during locomotion.
The ability to see objects clearly during locomotion is important for preventing collisions, falls, trips, and clumsy maneuvers. Clear vision requires that the image of the visual object of interest remains steady upon the retina. This ability to stabilize a retinal image is known to be compromised upon a change in g level after a prolonged exposure to a previous g level (for example, Earth return from a micro g orbit). To understand just why there is blurred vision in returning astronauts, it is necessary to understand jointly during locomotion: the motion of the head in space, the motion of the eye in the head and the relationship of the visual target to the person. Two important results from our research involve the relationship of head motion to walking speed and the relationship of eye motion to visual target distance.

Trunk and head movements were characterized over a wide range of walking speeds to determine the relationship between stride length, stepping frequency, vertical head translation, pitch rotation of the head, and pitch trunk rotation as a function of gait velocity. The results suggest that two mechanisms are utilized to maintain a stable head fixation distance over the optimal range of walking velocities. The relative contribution of each mechanism to head movement depends on the frequency of head movement and consequently on walking velocity. From consideration of the frequency characteristics of the compensatory head pitch, we infer that compensatory head pitch movements may be produced predominantly by the angular vestibulo-collic reflex (aVCR) at low walking speeds and by the linear vestibulo-collic reflex (lVCR) at the higher optimal speeds of walking.

Eye and head movements were also characterized during locomotion while the subject viewed near and distant targets. For near targets eye velocity was essentially in phase with head pitch velocity. Eye velocity increasingly lagged head pitch as target distance increased, and was compensatory at 2.0 m. For far targets, the gain and phase of eye *re* head pitch velocity indicated that the angular vestibulo-ocular reflex (aVOR) was generating the eye movement response. For near targets, decreasing target distance would augment the lVOR gain, and the eye velocity phase suggested that the linear vestibulo-ocular reflex (lVOR) was generating the vertical eye movement response.

The significance of the studies is that we have gained considerable insight into the compensatory and orienting functions of the vestibulo-collic and vestibulo-ocular reflexes during locomotion. Through this has come the development of countermeasure assessment criteria which can now be applied in studying behaviors that have proven to be difficult following exposure to microgravity.

2. Understanding the orientation mechanism during locomotion.

One of our working hypothesis was that the above-mentioned "profound impairments of posture, gaze and locomotion stability" are caused by alterations in compensatory and orientation mechanisms that are generated in the central vestibular system from motion inputs. During exposure to altered gravity, the motion inputs from the otolith organs are "distorted" compared to the on-earth conditions. These distortions, in turn, cause both inappropriate body head and eye movements and an altered sense of orientation, which degrades stability during locomotion.

We compared motions of the body during walking along a straight line with body motions while walking along a curved path. In the latter condition subjects accelerate in toward the direction of the curve which introduces an inertial component which may or may not effect measures of their body orientation in space. Our data show that compensatory eye, head and body movements stabilize gaze during straight walking, while orienting mechanisms direct the eyes, head and body to tilts of the resultant of gravitational and centripetal acceleration in space during turning. This finding in normal subjects can now be compared to subjects with known impairments in their balance system or to returning astronauts to determine whether or not such individuals can successfully align parts of their bodies in an appropriate way while turning.

3. Characterizing the recovery trajectory to disturbances of locomotion.

Analysis of perturbed gait provides a means of evaluating the success of measures designed to counter loss of balance due to disease or exposure to microgravity. We measured several body segment variables (head, sternum, legs) and especially their trajectories in response to mechanical perturbations that were precisely delivered in time, magnitude, and direction to the foot.

In normal individuals, the recovery trajectory show a large initial displacement due to the disturbance and subsequently crosses the baseline at the second step to show a slight underdamped response at the third step with a return to, or near, the baseline by the fourth step. In contrast, the recovery trajectory for a pilot vestibulopathic patient shows a distinctive different pattern which takes several more paces to recover.

The development of an experimental paradigm that introduces a calibrated disturbance to the foot during the support phase of normal locomotion provides a means for the objective quantification of locomotor response dynamics that are known to be altered in astronauts upon return from exposure to microgravity but for which no current test exists. Returning astronauts whose orientation mechanism has been distorted and patients having vestibulopathies that may well

affect their orientation mechanism are expected to have longer recovery trajectories than healthy normals.

Satisfaction of hypotheses, objectives, and specific aims of original proposal.
The first three of the original aims of this project and their hypotheses were successfully accomplished and produced the three key findings mentioned above. A fourth aim was truncated due to lack of a suitable patient population. A fifth aim was consolidated with an aim in another project. The final aim, which involved development of methodology, was integrated into the first three aims.

Implications for Critical Path Risk reduction and links to health research on Earth.
The results from this project apply directly to the Critical Path Risk of impaired neuromuscular strength upon return to positive G leading to increased occurrence of falls and fractures during emergency egress and escape – a Type II risk. We have defined some situations that occur in every day locomotion in which astronauts returning from microgravity and in which patients having subtle vestibulopathies are apt to have trouble but which there are no objective measures currently available to quantify their performance. From these, we have increased our understanding of some of the fundamental processes that govern factors like gaze and head position while moving. There is now the potential for developing more meaningful and sensitive tests of balance function that can be applied to astronauts for countermeasure assessment, and to patients with balance anomalies.

NSBRI RESEARCH PROGRAM
RADIATION EFFECTS

Team Leader: Dicello, J. F. Hopkins/SOM

Dicello, J. F.	**PI**	**Hopkins/SOM**	**Radiation Effects Core Project**	**103**
Cucinotta, F. A.	CO-I	NASA JSC		
Gridley, D. S.	CO-I	Loma Linda		
Howard, S. P.	CO-I	Wisconsin		
Huso, D. L.	CO-I	Hopkins/SOM		
Mann, J.	CO-I	Hopkins/SOM		
Moyers, M. F.	CO-I	Loma Linda		
Nachman, K.	CO-I	Hopkins/SOM		
Novak, G. R.	CO-I	Hopkins/SOM		
Piantadosi, S.	CO-I	Hopkins/SOM		
Ricart, R.	CO-I	Hopkins/SOM		
Simonson, D.	CO-I	Hopkins/SOM		
Strandberg, J. D.	CO-I	NCRR/NIH		
Yang, T.	CO-I	NASA JSC (Deceased)		
Huso, D.L.	**PI**	**Hopkins/SOM**	**Chemoprevention of Radiation-Induced Rat Mammary Neoplasms**	**105**
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Zhang, Y.	CO-I	Hopkins/SOM		
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RADIATION EFFECTS TEAM
PROGRAM EXECUTIVE SUMMARY

Radiations in space from galactic and solar sources are generally considered to be one of the three or four most serious hazards with regard to long-term human missions. A recent National Research CounciVNational Academy of Sciences (1996, 1998) report, for example, and an earlier related report (1996) discuss the significance and consequences of radiation exposures in space. The NASA Critical Path Roadmap (http://criticalpath.jsc.nasa.gov/main.asp) classifies Radiation Effects as one off our Type-I severe risks, those of most concern, along with Bone Loss, Human Behavior, and Clinical Capability. NASA's Critical Path Roadmap lists five major risks from radiation, categorizing them with "I" being the most significant risk type and "1" being the most significant risk rank:

Table 1. Potential consequences from radiation exposures in Space, according to the NASA Critical Path Roadmap.

Risk Factors	Risk Type	Risk Rank
Carcinogenesis	I	1
Damage to the central nervous system from radiation	II	2
Synergistic effects from radiation, microgravity, and other spacecraft environmental factors	II	3
Early and acute effects from radiation exposure	II	4
Radiation effects on fertility, sterility, and heredity	III	5

The National Space Biomedical Biomedical Research Institute (NSBRI) was formed to address the medical risks to humans in space as well as the subsequent risk to mission success. One of NASA's goals in supporting the institute is to understand the effects of space radiation on biological, chemical, and physical systems and processes. An essential strategy of the NSBRI has been the Integrated Research Team concept to maximized productivity and cost-efficiency with minimum redundancy to build and balance integrated research programs. A main mission of the NSBRI is to design and validate countermeasures addressing the major hazard. At the end of the first three years, the Radiation-Effects Team has become one of the most productive, cohesive teams focusing on the first four risk factors in Table 1 and one of the few to have shown feasibility of a proposed countermeasure, the use of chemopreventive agents or dietary supplements to reduce the risk of cancer from low-dose exposes to high atomic-number, energetic charged particles (HZEs) and protons.

The present Team organization, as outlined in the following figure, evolved from the original missions of the NSBRI and the Phase-I and Phase-II NSBRI proposals to NASA. The overall philosophy is that the Core Project provides the *in-vivo* results for risks of cancer and other

diseases and the Chemoprevention Project provides the data for risk reduction with Tamoxifen. The Cytogenetics Project gives us cellular and cytogenetic data for chromosome aberrations for cells irradiated in the animal or in-vitro and the DNA Project provides us with mutation data related to repeated sequences, in both cases to provide us mechanistic information for extrapolation to humans and parametric data and benchmarks for risk assessments. Finally, the Core Project assembles all of the data to calculate risks in the animal model and to extrapolate to risks of humans in space.

Almost every review of the radiation problems in space, including the three previous references, has recommended animal studies to quantify the risks to these types of radiation and to pursue likely countermeasures. Until this series of experiments, however, there had been only one comprehensive animal study to investigate the effects of ions of high atomic number and high energy, HZEs. That experiment was conducted by Alpen et al. (1993) with the Berkeley Bevalac, which has been out of commission for almost a decade. It has provided invaluable data on carcinogenesis in the harderian gland of a mouse model as a function of linear energy transfer (LET), and it has been a cornerstone for risk assessments in space during the last decade. No comparable series of experiments had been conducted to evaluate the use of drugs to reduce the risk of cancer from exposures in space.

As a result of the scientific reviews, meetings, and discussions during the developmental period of the NSBRI, the Core Project chose as its animal model the female Sprague-Dawley rat to be irradiated whole-body with HZEs, protons, or photons, and evaluated the biological consequences, including malignant and benign tumors at all sites, pituitary and CNS damage, and other diseases. The motives for this choice were multiple and are presented in detail in the Final Report for the Core Project; however, this model was that recommended by the members of the External Advisory Council. Animal experiments of this type were generally not done previously because of the complicated logistics and the large expensive. Only three facilities in the world, one at Brookhaven National Laboratory in New York State, one in Germany, and one in Japan, produced the necessary accelerator HZE beams. The costs for HZE beam time is millions of dollars a year, far in excess of any funds available from the NSBRJ, and only about 150 hours a year are available for all space-biology irradiations in the U.S.A. Finally, no one had ever carrying out experiments with energetic charged particles at multiple facilities, including Loma Linda University with its energetic proton synchrotron. The logistics of transporting thousands of animals between multiple facilities in isolated environments and keeping them alive subsequently for three or more years was at best a challenge. To maximize the value of the results, the team lead offered colleagues in the other projects to join forces to maximize the production of useful scientific data with the irradiated animals and to provide different information but correlated to the same animal species and to humans so that the results could be applied most efficiently to humans in the space environment. Three other projects that successfully survived the review process with two joining forces to use the Sprague-Dawley, one studying Tamoxifen (Howard/Huso) as a chemopreventing agent and the other to look at cytogenetics to predict cancer risks (Williams) and to correlate the Spraque-Dawley results to human mammary cells, human lymphocytes, and eight different human colorectal cell lines with varying status of p53 expression. The third project (Sinden) proposed originally to use low-energy helium microbeams to study repeated DNA sequences using techniques that were established at that time only for mouse models. During the intervening time, the principal investigator has redirected the project goals to study the more energetic iron beam at BNL and has been developing reporter constructs for the Spraque-Dawley rat.

At the end of the first funding cycle, the four projects have become almost indistinguishable in their goals and in their cooperation. That focused, cooperative effort has succeeded in implementation and execution of one of the most relevant but most difficult series of experiments performed as part of NASA's Life Sciences program in at least a decade.

At the end of the first three years of the team program, the investigators have designed, built, and successfully implemented systems to transport and irradiate both animals and cells. Three series of experiments were performed, each examining the consequences of 1-GeV iron ions, 250-MeV protons, and gamma rays from cesium-137 and cobalt-60, as well as sham irradiations. In each case, the animals were irradiated whole body at doses comparable to those expected in space. The animals are cared for and monitored daily, and all diseases are medically treated.

We now have statistically significant data for the risk for mammary fibroadenomas and adenocarinomas and for pituitary-related diseases as functions of particle type, dose, and time, as discussed at length in the project reports. In parallel with the animal experiments, we have been examining cell systems. The principal objectives of these projects were to examine cell survival, cytogenetic damage, and DNA deletions and recombinations understand the initial damage and the mechanisms responsible for the initial damage and the subsequent promotion and progression of the diseases. We are developing theoretical models to simulate the biological alterations and the in-vivo responses. We have used our data and models for preliminary calculations for the risk of cacinogenesis in the animals.

RESEARCH AREA:	Radiation Effects: DNA Damage and Repair
PRINCIPAL INVESTIGATOR:	John F. Dicello, Ph.D.
ORGANIZATION:	Johns Hopkins Oncology Center
PROJECT TITLE:	Radiation Effects: Core Project
FUNDING:	$352,111 (FY 1998); $571,006 (FY 1999); $1,136,716 (FY 2000)
TOTAL FUNDING:	$2,123,885

PROJECT EXECUTIVE SUMMARY

The risks to personnel in space from the naturally occurring radiations are generally considered to be one of the three or four most serious limitations to human space missions, as noted in two reports of the National Research Council/National Academy of Sciences (1996, 1998) and the NASA Critical Path Roadmap. The main objective of the Core Project of the Radiation Effects Team for the National Space Biomedical Research Institute is to study the consequences of radiations in space in order to develop countermeasures, both physical and pharmaceutical, to reduce the risks of cancer and other diseases associated with such exposures.

During interplanetary missions, personnel in space will be exposed to galactic cosmic rays, including high-energy protons and energetic ions with atomic masses of iron or higher. In addition, solar events will produce radiation fields of high intensity for short but irregular durations. The level of intensity of these radiations is considerably higher than that on Earth's surface, and the biological risks to astronauts is consequently increased, including increased risks of carcinogenesis and other diseases. Carcinogenesis from space radiation is one of the major health concerns for long term space travel. Protons are the most abundant component of the space radiation in both solar particle events and in the galactic cosmic rays. The corresponding dose-rates for proton exposure range from 10^{-3} to 0.1 Gy/hr, with possible accumulated doses of roughly 0.5-2 Gy in a large solar particle event or for interplanetary travel. These dose-rates are lower than those used in therapeutic treatment of cancers or encountered in epidemiological studies of A-bomb survivors.

This group is examining the risk of cancers resulting from low-dose, low-dose rate exposures of model systems to photons, protons, and iron by using ground-based accelerators which are capable of producing beams of protons, iron, and other heavy ions at energies comparable to those encountered in space. The specific aims of this work include *in-vivo* studies of carcinogenesis resulting from exposures to low doses of energetic heavy- ions, protons, and photons. We have successfully conducted a series of experiments using a 1-GeV iron beam at the Brookhaven National Laboratory and 250-MeV protons at Loma Linda University Medical Center's proton synchrotron facility. As part of these studies, this group is investigating the potential for the pharmaceutical, Tamoxifen, to reduce the risk of breast cancer in astronauts exposed to the level of doses and particle types expected in space. These data are essential for an improved evaluation of the cancer risks from radiation in space. Nevertheless, this is only the second large-scale study of this type and only the first including a study of a chemopreventive agent. Although the experiments are only in the preliminary stages, extensive data have been forthcoming and are reviewed in this report.

Theoretical studies are being carried out as part of this project in a collaboration between scientists at NASA's Johnson Space Center and Johns Hopkins University. The theoretical studies, in coordination with the experimental program, have provided methods and predictions

which are being used to improve our evaluation of radiation risks to be encountered and to evaluate appropriate strategies for countermeasures. Continued collection and analysis of data from this project over the next three years will enhance the precision of our estimates of biologic response and reduce the unacceptable uncertainties associated with present risk assessments of activities in space which increase vulnerability and costs.

Although the work in this project is primarily directed toward problems associated with space travel, the problem of protracted exposures to low-levels of radiation is one of national interest in our energy and defense programs, and the results may suggest new paradigms for addressing such risks.

RESEARCH AREA:	Radiation Effects: DNA Damage and Repair
PRINCIPAL INVESTIGATOR:	David L. Huso, DVM, Ph.D. (Replacing S. Howard)
ORGANIZATION:	Johns Hopkins Oncology Center
PROJECT TITLE:	Chemoprevention of Radiation Induced Rat Mammary Neoplasms
FUNDING:	$122,894 (FY 1998); $106,912 (FY 1999); $152,578 (FY 2000)
TOTAL FUNDING:	$338,332

PROJECT EXECUTIVE SUMMARY

Chemoprevention is a pharmaceutical approach to arresting or reversing the process of carcinogenesis during cancer's typically prolonged latent period (often 20 years or more) before invasion or metastasis occurs. Surging scientific and public interest in applying chemoprevention strategies to people in the general population that have been identified to carry even slight increases in the risk of developing cancer (e.g. genetic risk) is fueling the identification of exciting new chemopreventive agents. Some now argue that future development of chemopreventive agents offers greater potential for the long-term control of cancer than the much more widely studied and aggressively pursued chemotherapy agents. The basis for this optimism is seen in ongoing investigations that continue to reveal the step by step genetic and molecular basis of cancer development (especially early events). The emerging knowledge of the molecular mechanisms of cancer provides potential targets for specific agents that allow rational approaches to be devised for the chemoprevention of cancers.

The major long-term risk associated with radiation exposure during space travel is predicted to be radiation-induced cancer. The cancer-causing effects of low-LET radiations such as x-rays, g-rays, or electrons, typical of environmental earth exposures, have been relatively well-established. However, radiation likely to be encountered in space includes mainly heavy ions and protons along with their secondaries. Much less is known about the biology and risks associated with these types of radiation. The doses of radiation likely to be received even for long missions are probably low, but cover a broad range and are very unpredictable due to solar events. Like other types of radiation, the increased cancer risk associated with proton and heavy ion exposure is troubling because many radiation-induced cancers do not appear until later in life. Therefore, a large amount of uncertainty exists in how best to assess and manage the radiation risks associated with space travel.

Two high priorities in preparation for long missions are 1) providing a better understanding of both the short-term and long-term carcinogenic effects of heavy ion or proton radiation and 2) developing pharmaceutical countermeasures to mitigate the carcinogenic risk associated with low-dose and mid-dose exposures to these types of radiation.

As countermeasures to the cancer risk associated with space travel, chemoprevention offers a particularly promising avenue for investigation because of: 1) the difficulties associated with absolutely blocking radiation-induced mutagenic damage to DNA during prolonged space travel, either with shielding or pharmaceuticals, and 2) the prolonged latency period of most radiation-induced cancers (especially at low doses). This offers a prolonged time period when the most successful chemopreventatives exert their effects. For most cancers, compounds that modulate the regulation of cell growth and apoptosis (rather than blocking mutagenic damage to DNA) have to date shown particular promise in preventing overt cancer from developing in susceptible organs. Current chemoprevention successes against sporadic or familial tumors have identified

tamoxifen for prevention of breast cancer, NSAID's (nonsteroidal antiinflamatory drugs) for prevention of colorectal cancer, and retinoids for preventing oropharangeal and other cancers as currently the top candidates for successful chemopreventation of specific tumors in humans.

Organs are not equally sensitive to the carcinogenic effects of radiation. Tissues that appear to be at higher risk for developing radiation-induced neoplasms include the female breast, the gastrointestinal tract (colorectal cancer), the thyroid, the bone marrow/lymphoid system (leukemia), and the lung. The female breast is particularly sensitive to the carcinogenic effects of radiation and therefore a relevant tissue in which to study chemoprevention of radiation-induced cancer. Furthermore, one of the most important advances in the therapy of breast cancer in the past two decades has been the development and use of tamoxifen for breast cancer chemotherapy. Over the past few years, tamoxifen has also emerged as an effective chemopreventative and now is the most widely prescribed anticancer drug in the world. It is prescribed mainly for breast cancer.

The class of compounds that includes tamoxifen, the selective estrogen receptor modulators (SERM's), are thought to have outstanding potential for use in estrogen replacement therapy and as chemopreventive agents. Burgeoning research and development of new SERM compounds has led to many new and improved SERM's undergoing trials. Tamoxifen, however, remains the prototype SERM for breast cancer chemoprevention. Newer SERM's will hopefully further improve on tamoxifen's effects while reducing its side effects. SERM's are ligands for the estrogen receptor (ER) and modify carcinogenesis in breast epithelial cells by antagonizing ER signaling. However, in other tissues SERM's can act as partial ER agonists and promote the beneficial effects of estrogens in, for example, the skeletal and cardiovascular systems. Interestingly, tamoxifen may also affect carcinogenesis in a number of organ systems by disrupting apoptosis regulation in proliferating cells. In spite of the widespread use of tamoxifen, very little is known about its lifetime effectiveness against radiation-induced neoplasms-particularly those induced by radiation likely to be encountered in space such as protons and heavy ions.

Appropriate animal models provide a powerful means for directly evaluating the effectiveness of particularly promising chemopreventatives against cancers that may occur following radiation exposure. The rat mammary tumor model has been used extensively to analyze the carcinogenic effects of both chemical xenobiotics and physical agents. The Sprague Dawley rat mammary tumor model is particularly well-suited for studies in the low dose range because it is prone to develop mammary neoplasms early in life. Previous studies using the Sprague Dawley model have shown that sublethal doses of radiation (xrays, gamma rays, neutrons-not particularly relevant to space travel) induced mammary tumors, often within one year, and with a linear dose-effect relationship. Thus the Sprague Dawley rat mammary carcinogensis model not only closely resembles human breast cancer biologically, but it also is a highly sensitive model in which to examine the effects of radiation exposure and for testing pharmaceutical countermeasures against radiation effects. Our initial studies have focused on the effects of whole body, low level heavy ion and proton radiation along with chemoprevention of similarly induced mammary tumors using the female Sprague-Dawley rat mammary tumor model. Our rational approach to chemoprevention (SERM's) is based on one of the few successful emerging chemoprevention strategies used in human cancers to date in regard to sporadic or familial neoplasms. The well-studied, widely prescribed, protoypte SERM, tamoxifen has been effectively and safely used in humans for chemotherapy for almost two decades. These advantages, along with an understanding of its molecular mechanism of action suggests it would be an excellent candidate for successful long-term chemoprevention of specific proton and heavy ion-induced cancers. The

prospect for successful long-term chemoprevention of this potentially important, late-appearing cancer relevant to space radiation exposure is indeed an exciting prospect.

Our hypothesis is:
If there is an increased risk for developing cancer due to radiation exposure during prolonged space travel, the increased cancer risk can be mitigated by chemopreventive countermeasures implemented during the long cancer latency period that follows radiation exposure. A logical and relevant area in which to test this hypothesis is in a radiation-induced breast cancer animal model since the female breast is one of the tissues most sensitive to the cancer-inducing effects of radiation and because recent evidence suggests that the compound tamoxifen is an effective breast cancer chemopreventative.

Our key findings thus far are:
Dr. Huso took over as PI of the chemoprevention studies less than two years ago and since that time considerable progress has been made in this area. Our studies are not complete, but preliminary evidence suggests that tamoxifen will be highly effective in preventing at least the mammary carcinomas that appear early following photon, proton, and heavy ion radiation exposure in the mammary gland. However, it appears there may be some variation in effectiveness depending on the dose and quality of radiation to which the individual is exposed. In addition, it is important to complete these studies and determine the effectiveness of tamoxifen for long-term chemoprevention of radiation-induced mammary cancer.

Although it is still early in the studies, preliminary results from our ongoing tamoxifen studies have pointed to a proof of principle for a strategy in which chemopreventive agents could play an important role in preventing breast cancer following exposure to radiation during space travel. This suggests that new chemopreventatives could be similarly identified that prevent other specific cancers associated with proton and heavy ion radiation exposure relevant to space exploration. Since cancer chemoprevention in general is still in its infancy as an emerging field, chemoprevention based on new targets and emerging compounds, hold considerable promise for continued improvement of strategies to effectively mitigate risks associated with radiation and other predisposing factors for cancers. Further studies are required to confirm the long-term safety and effectiveness of chemoprevention strategies, to identify additional agents that are effective against specific neoplasms, and to continue to improve chemoprevention effectiveness and implementation.

The implications of our findings for risk reduction for both space exploration as well as for the general population:
The implications are clear. Our results, though preliminary, provide a glimpse of the enormous potential payoff that chemoprevention research could provide in the battle against cancer. Regardless of the reason for an individual to be at increased risk for developing particular cancers, be it radiation exposure as in our studies (relevant to space travel) or genetic and enviornmental factors (relevant to the general population), specific chemopreventive compounds and strategies can be identified and implemented to mitigate risks that predispose individuals to cancer. Much work remains to be done to fully realize the benefits of chemoprevention strategies in the battle against cancer. Support for research into chemoprevention of radiation-induced neoplasms such as that provided by NSBRI therefore benefits not only space exploration efforts, but what is learned in this important area also could provide unique insight into cancer chemoprevention for the general population.

RESEARCH AREA:	**Radiation Effects: DNA Damage and Repair**
PRINCIPAL INVESTIGATOR:	**Jerry R. Williams, Sc.D.**
ORGANIZATION:	**Johns Hopkins Oncology Center**
PROJECT TITLE:	**Radiation-Induced Cytogenetic Damage as a Predictor of Cancer Risk for Protons and Fe Ions**
FUNDING:	**$200,000 (FY 1998); $205,616 (FY 1999); $205,616 (FY 2000)**
TOTAL FUNDING:	**$611,232**

PROJECT EXECUTIVE SUMMARY

This project has made several key findings that describe, in detail, chromosomal damage induced by three model space radiations: photons, energetic protons and energetic iron-ions (Fe-ions). The large number of data produced provide new insights into the relative potency of these radiations to induce different types of aberrations in multiple cell types: human lymphocytes, human mammary cells, rat mammary cells (in vivo and in vitro) and multiple forms of human colorectal tumor cells that have specific modulations of cancer-relevant genes. Further, we have investigated the induction of these radiations when used in fractionated or protracted time patterns in human lymphocytes, these data providing a paradigm for extrapolation of data from acute exposure to other exposure patterns. Further, the analysis of multiple aberrations per cell may be a "signature" for the dose and quality of radiation that induced these damages. We observe specific changes in induction of chromosome aberrations in cells that are deficient in expression of p53, p21 and 14-3-3-sigma, demonstrating that early changes in cells may render them more susceptible to further genetic damage induced by the three model space radiations. These data also provide new insights into the mechanisms of molecular biology by which cells process radiation damage. We have also measured clustering of multiple aberrations in individual cells so that Poisson analysis can be used to consider the relative influence of radiation quality on multiple events. Finally, we have suggested a new model for the dose response of cells to these radiations, focusing on induced cellular processing of radiation damage. Our model, that we term the subalpha- alpha- omega (SAO) model, provides a new structure for mathematical paradigms for testing whether chromosome aberrations can be used as a surrogate marker in estimating cancer risk. Our data on chromosome aberrations, when combined with the outcome of parallel studies in carcinogenesis in the Sprague-Dawley Rat, will provide a direct comparison between the rate of induction of chromosome aberrations in mammary epithelial cells with the rate of induction of mammary cancer in the same animal model over the same dose-ranges.

RESEARCH AREA:	**Radiation Effects: DNA Damage and Repair**
PRINCIPAL INVESTIGATOR:	**Richard R. Sinden, Ph.D.**
ORGANIZATION:	**Texas A&M University**
PROJECT TITLE:	**Quantitation of Radiation Induced Deletion and Recombination Events Associated with Repeated DNA Sequences**
FUNDING:	**$50,000 (FY 1998); $60,000 (FY 1999); $50,000 (FY 2000)**
TOTAL FUNDING:	**$160,000**

PROJECT EXECUTIVE SUMMARY

Background and Significance of the Project:

Effects of Radiation on Biological Systems

Manned exploration of space exposes the explorers to a complex and novel radiation environment. The galactic cosmic ray and trapped belt radiation (predominantly proton) components of this environment are relatively constant, and the variations with the solar cycle are well understood and predictable. The level of radiation encountered in low earth orbits is determined by several factors, including altitude, inclination of orbit with respect to the equator, and spacecraft shielding. At higher altitudes, and on a Mars mission, the level of radiation exposure will increase significantly. A significant fraction of the dose may be delivered by solar particle events which vary dramatically in dose rate and incident particle spectrum. High-LET radiation is of particular concern. High-LET radiation, a component of galactic cosmic rays (GCR), is comprised of a variety of charged particles of various energies (10 MeV n^{-1} to 10 Gev n^{-1}), including about 87% photons, 12% helium ions, and heavy ions (including iron).

These high energy particles can cause significant damage to target cells. The different particle types and energies result in different patterns of energy deposition at the molecular and cellular level in a primary target cell. They can also cause significant damage to other, nearby cells as a result of secondary particles. Protons, for instance produce secondaries that include photons, neutrons, pions, heavy particles, as well as gamma rays. Heavy ions deposit energy in a "track" in which the magnitude of the damage varies as the particle loses energy. Heavy ions produce secondary delta rays, or electrons. The distribution of damage through tissue is described by a Bragg curve which will be characteristic for different energies. Needless to say there are differences in the RBE of protons and α particles.

High-LET heavy ions are particularly damaging to cells as they do continual damage throughout their track. Differences in these energy deposition patterns can significantly influence the nature of DNA damage and the ability of cellular systems to repair such damage. It has been suspected that these differences also affect the spatial distribution of damage within the DNA of the interphase cell nucleus and produce corresponding differences in endpoints related to health effects. The interaction of a single high-LET particle with chromatin has been suggested to cause multiple double strand breaks within a relatively short distance. In part this is due to the organization of DNA into chromatin fibers in which distant regions of the DNA helix can be physically juxtaposed by the various levels of coiling of the DNA. This prediction was confirmed by the detection of the generation of double strand DNA fragments of 100-2000 bp following exposure to high-LET ions (including iron).

While it is very clear that ionizing radiation can cause cytogenetic damage and cancer, relatively little is yet known about the mutagenic or carcinogenic effects of high energy HZE particles in

cells. High-LET radiation produces proportionally more double-strand than single strand breaks compared with low-LET radiation. Double-strand breaks are likely responsible for the cytogenetic damage visible as chromosomal aberrations, transformation, mutations, and delayed cell death.

Nearly one-third of the human genome is composed of DNA repeats, which include simple mono-, di-, tri-, and tetranucleotide repeats; widely separated small and large repeats; and inverted repeats. Mutations associated with repetitive DNA are a source of many genetic diseases and cancer. Therefore, understanding how the various kinds of repeats contribute to the disease burden and understanding the impact of DNA damage on repeat-associated genomic instability is important for human health. Such repeated DNA sequences are likely to be very prone to mutation following exposure to high-Z high-energy (HZE) particles during space flight. Cells in the direct line of the HZE particle sustain a high dose of energy while cells surrounding the primary tract sustain a lower dose of energy from the energetic delta rays (electrons) produced by HZE particles. Therefore, the nature and pattern DNA damage to cells in tissue upon irradiation with HZE particles is particularly complex. It is important to understand the types of mutational changes induced by both the HZE particles as well as the delta rays.

Given the high frequency of occurrence of repeated DNA sequences it is highly likely breaks or base damage from radiation will occur within these sequences. Moreover certain processes of repair and recombination involve the generation of free 3' ends in DNA and extended single-strand regions that expose repeats to recombination or primer template misalignments. Therefore, the molecular events that are responsible for cytogenetic damage (chromosomal breaks and rearrangements) and other mutations (point mutations, frameshifts, small deletions and duplications) in many cases will involve primer template misalignments. (Note that we have shown that the molecular mechanism for a hotspot for several +1 frameshift mutations involves intermolecular strand switch events (primer template misalignments) that occur specifically in during leading strand replication at a region containing DNA repeats (Rosche et al., 1998).) It is also possible that a cell sustaining substantial damage from a heavy iron particle hit may saturate some or all of its repair capability, or induce an error prone mode of repair to mediate survival. The types of assays we are developing will provide sensitive reporters of the replication/repair fidelity of a cell following damage from HZE particles. It is the fidelity of this process which, if compromised, will ultimately lead to carcinogenesis or other detrimental effects of radiation damage. With the sensitive reporter constructs we are developing, the protective effects of chemopreventive measures or countermeasures can be quickly established. A major goal of this project was to provide a rapid way to test the efficacy of various countermeasures and chemopreventive drugs with respect to mutation minimization and cancer prevention. The sensitive, relative rapid assay being developed here would compliment the long-term Dicello rat study being conducted at Johns Hopkins.

Goal:

The goal of this proposal was to develop data on the relationship between gene mutations, including deletions and recombination associated with direct repeats, and the quantity and quality of the radiation that interacts with the biological system so that countermeasures designed to minimize the health risks of radiation exposure in space can be devised. This goal could be accomplished by quantifying the rate of deletions between direct repeats, which may involve primer-template misalignment, recombination, or gene conversion in human cells following exposure to radiations which reproduce the energy deposition patterns produced in individual cells by the radiation environment in space. Using cell lines that provide sensitive reporters of mutations involving deletions between direct repeats and recombination events, we measured the

rate of mutations in irradiated cells and in progeny of irradiated cells, following exposure to high energy alpha particles. We also planned to analyze several biological endpoints in other cells lines that do not contain genetically selectable end points, but which contain long tracts of direct repeats (1.8 mb) or inverted repeats (15.3 kb). In addition, we also began developing additional reporter constructs for application in Sprague-Dawley rat mammary cells (and eventually rate) to increase the sensitivity of measuring deletions and recombination events mediated by DNA repeats. This will complement the long-term rat carcinogenesis study of the Radiation Effects Group, by providing a rapid, sensitive screen for the effects of chemopreventive and radioprotective drugs on genome instability following exposure to HZE particles and protons.

Hypothesis:
The hypothesis driving this proposal is that DNA damage introduced by high-energy (HZE) particles induces aberrant DNA repair events, involving repeated DNA sequences that lead to recombination, gene conversion, or other mutation, that initiate the sequence of cytogenetic and functional changes which manifest themselves as the long term health effects of radiation exposure in space, including cancer. Knowing the types of mutational events induced by different radiations will contribute to sound decisions for optimizing shielding and reducing biological consequences through use of radioprotective drugs or various countermeasures. The cell lines and procedures utilized in this proposal will be useful for testing the efficacy of various countermeasures and chemopreventive drugs.

Brief Summary of the Key Results to Date:
The survival of four reporter cell lines (122-2, F14C-23, 7#7-7, and 3134) following exposure to 250 KeV X-rays has been measured. All cell lines were sensitive to X-rays in a range that would allow them to be used as reporter cell lines for radiation damage.

- The frequency of deletion of an inverted repeat and a nonpalindromic sequence from a *neo* gene in human 122-2 and F14C-23 cells have been measured (by isolating clones resistant to the antibiotic G-418) following exposure to 250 KeV X-rays.
- The nature of the reversion events, which involve precise deletions between direct repeats, has been analyzed by PCR analysis.
- The rate of reversion or the mutant frequency for the deletion mutations in the *neo* gene have been calculated for control and X-ray exposed cells. The frequencies are about 1-2 x 10^{-7} in sham (non irradiated) cells. The rate increases by as much as a factor of 60 following exposure to X-rays.
- The survival and G-418 reversion frequencies following exposure to 1000 MeV Fe particles at the BNL4 and BNL5 runs have been measured. Following Fe exposure, the rate of G-418 reversion increases as much as a factor of 100.
- We have successfully cloned a 770 bp perfect inverted repeat (2 x 385 bp Alu sequence) and a 763 bp inverted repeat with a 39 bp nonpalindromic center (2 x 362 bp + 39 bp) in *E. coli*. This has taken considerable effort as this is 6-7 times longer than any inverted repeat we have previously cloned. A number of modifications to existing protocols had to be developed to get this. We are putting *Sph*I adaptors on it to clone it into the *neo* gene in pJJ999 (the vector be used for electroporation into rat cells.

Progress Toward Testing the Hypothesis and Fulfilling the Goals of the Project:
We have made good progress toward testing the hypothesis. However, this project lacked sufficient funds to make the kind of progress necessary to obtain its ultimate goals. Progress was slow due to the lack of sufficient personnel to devote full time effort toward all aims. Given the financial limitations, I feel we made excellent progress. We were able to confirm that the experiments we designed would work. This was evident from our results from the BNL4 and

BNL5 radiations. Unfortunately, the pre-existing cell lines containing reporter constructs were not optimal for these experiments. Nevertheless, we were able to get the system to work and we correctly estimated the correct exposures for these experiments. Moreover, we learned the conditions necessary for the radiations.

Unfortunately, the project has come to an end just as we were at the verge of obtaining new reporter constructs (with the *alu* inverted repeats) that should work quite well for these experiments. More research and development was necessary in this area that was originally anticipated. The constraint of minimal personnel, slowed progress in this area.

Implications of the Results of this Project for Risk Reduction Related to the Critical Path and for Future Research on Earth Medical Problems:
This project was directed toward *understanding the molecular mechanisms of radiation damage and repair in cells.* These studies were designed to determine the relationship between the energy deposition pattern of radiation and its ability to increase the frequency of specific mutation events. DNA repeats are involved in many deletions and rearrangements associated with human disease. The reporter constructs, cell lines, and procedures to have been developed in this proposal will be directly applicable to studies on the effect of radioprotective drugs, as they would provide a very sensitive and relatively rapid quantitative assay for their effects. Moreover, these reporter constructs can be introduced into other types of cells and transgenic animals, including the Sprague Dawley rat. Thus, integration into the Dicello rat study may provide a way for ascertaining the effects of radiation and the protective effects of countermeasures, in a fraction of the time required for a long-term rat study. (Note, however, that the rat study must be completed for integration with a more rapid screen for countermeasures.)

Eventually our results will be useful for understanding the *genetic predisposition* to disease and cancer from radiation, which will be important for the potential genetic screening of astronauts.

Many other questions can be addressed with further application of our system, including those related to: chemical & biological agents that might be implemented to mitigate acute exposures, efficacy of radioprotectants, questions of shielding effectiveness, questions of fluence and fluence rate effects, and efficacy of nutritional supplements.

NSBRI RESEARCH PROGRAM
TECHNOLOGY DEVELOPMENT

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Kinnison, J. D.	CO-I	Hopkins/APL		
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TECHNOLOGY DEVELOPMENT TEAM PROGRAM EXECUTIVE SUMMARY

The objective of the Technology Development Program of the National Space Biomedical Research Institute is to develop systems, instrumentation, devices, algorithms, etc., that are important to the work of the other Research Teams in the Institute and the at-large space life science community. The unique feature of the program's effort is the opportunity to bring an integrated engineering systems perspective to bear on technological developments to support basic research. Multi-disciplinary development teams have been established to work on strategically focused projects that integrate individuals with vastly different capabilities into a cohesive team.

Four development projects were selected, by independent review, for pursuit under the technology development program. All projects demonstrated excellent progress in achieving their individual goals and objectives. To preclude unexpected technology issues and assure that the projects would meet the needs of the other Research Teams, rigorous design reviews were conducted during the first year. Each project team was also encouraged to work closely with the specific Research Team what would benefit from their development. Designs have all been completed and prototype development accomplished with sufficient application to real problems to demonstrate utility. This portends ultimate success for the projects, though definitive science results are pending.

The Compact, High Precision, Multiple Projection DXA Scanner project developed a concept for a low mass, volume, and power, high accuracy dual x-ray absorptiometer that will afford the ability to measure bone mineral density and geometry of the whole human body in space. This development supports the explicit needs of the Bone Demineralization/Calcium Metabolism Team. Acceleration of age related osteoporosis is rated as Risk Rank 1 of Risk Type I and the loss of skeletal muscle is rated at Risk Rank 1 of Risk Type II in NASA's Critical Path Roadmap. The project team has completed construction of a clinical engineering model and three-dimensional images of bone were successfully acquired. This device has greater precision and higher resolution than the latest commercial devices by at least a factor of three. It also has the ability to determine body composition of soft tissues important for the research of the Muscle Alterations and Atrophy Research Team, although this was a secondary objective. The value of this development is that a clinical engineering model has demonstrated the exceptional capabilities needed and that a flight instrument should have a mass of about 46 kg, tolerable by Space Station standards for a facility instrument. For the first time, the loss of bone mass, changes in bone geometry, and changes in body composition can be obtained throughout the whole body as a function of time during spaceflight. Whole body measurements are important because of the relatively poor correlation obtained between sites for age related osteoporosis and that bone loss in space may have different correlations. This will provide a better understanding of the basic processes at work, help assess the value of different countermeasures, and provide a method to mediate the application of the countermeasures eventually selected. System requirements for high-spatial resolution and rotational-scan geometry permits the system to provide the additional in-flight capabilities of digital radiography and x-ray computed tomography. The design is suitable for use in bed rest studies, the Space Station, and potential planetary missions. There has been extensive collaboration with the Bone Demineralization/Calcium Metabolism Team and one of the Co-investigators of this project is an Investigator on that team.

The Instrumentation for Non-Invasive Assessment of Cardiovascular Regulation project developed instrumentation to non-invasively apply cardiovascular systems identification (CSI) to identify mechanisms responsible for cardiovascular regulation and alterations. This project directly addresses the needs of the Cardiovascular Alterations Research Team and may be used to support studies conducted under other team protocols. Impaired cardiac response is rated as Risk Rank 1 of Risk Type II in NASA's Critical Path Roadmap. The project team completed the development of an engineering prototype system that had been configured with data acquisition and processing applications. The final goal to automate all system operations has been accomplished and CSI utility has been demonstrated on human test subjects. The software developed in C++ uses the Windows NT 4.0 operating system on a Pentium II 300 MHz Thinkmate computer. This is a powerful approach to identify mechanisms responsible for orthostatic intolerance and to provide quantification of the baroreflex, autonomic function, and other physiologic control mechanisms under varying environmental conditions, such as microgravity. It can be used in space and has already had extensive application to human subjects on Earth. Other teams (Bone Demineralization/Calcium Metabolism Team, Neurovestibular Team and Human Performance Factors, Sleep and Chronobiology Team) may use the technique to measure changes in autonomic nervous function. In addition, this project has many applications to important problems in clinical medicine such the diagnosis and management of patients with diabetic autonomic neuropathy, heart failure, and hypertension. There is close collaboration as the Principal Investigator is team leader and a Principal Investigator in the Cardiovascular Alterations Research Team. The Cardiovascular Alterations Research Team anticipates using this instrument in the study of astronauts before and after spaceflight and ultimately, during spaceflight.

The Miniature Time-of-Flight Spectrometer project adapted a high resolution, portable time-of-flight mass spectrometer for quantitative measurement of human biomarker compounds in space flight. Applications include analysis of breadth, body fluids, products of infection, and perhaps DNA repair products and DNA mutations. As currently configured the system is of special value to the Bone Demineralization/Calcium Metabolism and the Muscle Alterations and Atrophy Teams but biomarkers important to several other Research Teams can also be obtained. Acceleration of age related osteoporosis is rated as Risk Rank 1 of Risk Type I and the loss of skeletal muscle is rated at Risk Rank 1 if Risk Type II in NASA's Critical Path Roadmap. The flight device will be inherently rugged, have mass of less that 5 kg, and will require less than 50 Watts. Identification of compounds with mass ranges of from 100 to 10,000 amu has been demonstrated. Key elements of this project were the development of a reliable sampling method and the quantitation of the measurements. The team has demonstrated the ability to routinely detect and accurately quantify (1-2%) 3-methylhistidine in urine, insuline-like growth factors (IGF-1), and estradiol. Membrane and electro-spray sample collection processes have been demonstrated. There has been close collaboration with the Bone Demineralization/Calcium Metabolism and the Muscle Alterations and Atrophy Teams.

The In-Situ Spectrometry of Neutrons project developed a portable, real-time neutron spectrometer, for the range of 10 KeV to 500 MeV, to support the needs of the Radiation Effects/DNA Damage and Repair Research Team. Carcinogenesis caused by radiation is rated as Risk Rank 1 of Risk Type I in NASA's Critical Path Roadmap. The real-time neutron spectrometer prototype instrument is of portable brief case size, with a mass of less than 10 kg. Over the energy range of importance, it provides an energy resolution of 10 percent and also the counts for neutrons below 10 KeV. An alarm is included to warn astronauts when preset thresholds are exceeded. The project team has completed development of several test subsystems and an engineering model of the system, which includes two detector subsystems. The test

subsystems have been submitted to characterization tests carried out with alpha particles at APL and with neutrons at different energies at Clemson University, Columbia University, and the National Institute of Science and Technology. The engineering model was reconfigured and has been evaluated on a high-altitude aircraft test flight at NASA Dryden funded by a small synergy grant. The engineering model performed as expected to an altitude where a short in the high voltage, due to a corona breakdown, terminated data collection. There has been close collaboration with the Radiation/DNA Effects Research Team and a Co-Investigator is a Principal Investigator in that team. Based on the accomplishments achieved in this project, the research team was awarded two NASA grants to use the instrument to characterize materials to minimize the production of secondary neutrons and to provide a neutron spectrometer for a flight to Mars to determine the safety of the Martian surface.

A Technology Development Working Group, composed of representatives from the other Research Teams, monitored and established technology development needs. The working group participated in teleconferences, team site reviews, and the integration of a technology requirements document (available under separate cover). This widely distributed document served to identify the needs of the Research Teams to a wide audience to help assure a broad and appropriate response to NSBRI calls for proposals to the specific needs of the NSBRI Research Teams.

The Technology Development Team embodies a sense of synergy that is unique to the Institute. There is a cohesiveness that exists between the individual project teams and researchers within other Research Teams. As well, there is a strong intra-team coalition that enables free and open technology interchange. All of these attributes provide a strong basis for contribution to, and support of, the Institute's mission. To assure synergy, there were 23 teleconferences, meetings with other Research Teams, and, in the last year, monthly lunches for the Baltimore/Washington Investigators.

RESEARCH AREA:	**Technology Development**
PRINCIPAL INVESTIGATOR:	**Harry K. Charles, Jr., Ph.D.**
ORGANIZATION:	**Johns Hopkins University Applied Physics Laboratory**
PROJECT TITLE:	**Miniature X-ray Bone Densitometer**
FUNDING:	**$310,000 (FY 1998); $317,440 (FY 1999); $317,440 (FY 2000)**
TOTAL FUNDING:	**$944,880**

PROJECT EXECUTIVE SUMMARY

The purpose of the Advanced Multiple Projection Dual Energy X-ray Absorptiometry (AMPDXA) Scanning System project is to design, build, and test a precision scanner system for monitoring the deleterious effects of weightlessness on the human musculoskeletal system during prolonged spaceflight. The instrument uses dual energy X-ray absorptiometry (DXA) principles and is designed to measure bone mineral density (BMD), decompose soft tissue into fat and muscle, and derive structural properties (cross-sections, moments of inertia). Such data permits assessment of microgravity effects on bone and muscle and the associated fracture risk upon returning to planetary gravity levels. Multiple projections, coupled with axial translation, provide three-dimensional (3-D) geometric properties suitable for accurate structural analysis. This structural analysis coupled with bone models and estimated loads defines the fracture risk. The scanner will be designed to minimize volume and mass (46 kg goal), while maintaining the required mechanical stability for high-precision measurement. The AMPDXA will be able to detect 1% changes in bone mass and geometry and 5% changes in muscle mass.

The development of the AMPDXA is being carried out in a multistage process beginning with an initial Laboratory Test Bed to prove basic principles and develop initial hardware and software. Following the Laboratory Test Bed was a Clinical Test System, which is now operational, which will allow the testing of AMPDXA principles on human subjects as well as on special phantoms and other test objects. The third stage is the design and development of a protoflight system that incorporates all the design and performance features of the previous units into a form, fit and functional configuration that could evolve into the low mass flight unit. Preliminary designs for this protoflight system incorporate advanced electronic fabrication technologies (chip-on-board, multichip modules) coupled with commercial (off-the-shelf) parts to produce a reliable, integrated system which not only minimizes size and weight, but because of its relative simplicity, is also cost effective to build and maintain. Additionally, the protoflight system is being designed to minimize power consumption. Methods of heat dissipation and mechanical stowage (for the unit when not in use) are being optimized for the space environment.

The AMPDXA Project is a joint effort between the NSBRI's Technology Development Team and both the Bone Demineralization/Calcium Metabolism Team and the Muscle Alterations and Atrophy Team. Its goal is to provide the high precision monitoring system necessary to fully assess both the deleterious effects of weightlessness on the bones and muscles and the effectiveness of any countermeasures. We believe that any pharmacological or exercise-related countermeasures used by astronauts to mitigate microgravity effects will require efficient and timely monitoring. Moreover, the monitoring device must be capable of being used by astronauts during spaceflight so that feedback can be dynamically employed to regulate countermeasure doses. The system design will be such that intelligent but not necessarily medically trained personnel will be able to create scans that will provide all of the accuracy and precision necessary. Readouts and displays for the AMPDXA instrumentation will be specifically

designed to provide useful (real-time) feedback information to both the astronauts and the ground-based physician monitoring team (as permitted by the mission dynamics).

We believe the key to understanding the mechanism of bone (and muscle) loss in space (microgravity) lies in the bone's structural details and the changes in the structure due to prolonged weightlessness. Our hypothesis is that throughout most of adult life, aging bones become more structurally efficient and retain their strength even though BMD declines. The homeostatic mechanism for strength maintenance depends on skeletal loading. Thus, to maintain bone strength, normal loading on the skeletal system must be maintained. Absence of loading during prolonged spaceflight (or disuse) can cause uncompensated loss of bone strength. Even reduced loading (caused by muscle wasting and inactivity in the elderly) can cause a disruption in the bone strength maintenance mechanism.

Current bone and muscle mass measurements (via conventional DXA or ultrasound) are regional averages that obscure structural details. Since the mechanical consequences of lost bone and muscle are reflected in the structure, an absolute determination of skeletal mechanical competence is needed to supplement the loss measurements. Engineering properties of the bones can be derived from DXA-generated BMD data. Our method derives geometrical measurements from the BMD images. From such images, we extract BMD profiles at important skeletal locations (e.g., proximal shaft and femoral neck). Key properties measured and derived from these profiles include the BMD, the subperiosteal width, the section modulus (related to strength), and the cortical dimensions.

During the course of the AMPDXA Project, significant progress has been made in several key areas: (1) instrument development, (2) algorithm development for BMD image extraction and structural analysis, and (3) bone reconstruction and modeling techniques. As mentioned above, a full size Laboratory Test Bed (1 meter source to detector distance) was constructed to verify principles and theoretical predictions. Scanning is provided by high-precision rotation and translating stages. This Laboratory Test Bed, in conjunction with a high-resolution detector and our analysis software, has produced some exciting preliminary results. The improvement in spatial and contrast resolution with our scanner is quite evident.

The next important instrumentation step has been the development of a Clinical Test System. The Clinical Test System incorporates a high precision rotation and translation stage to provide the scanning capability to carry out qualification tests on human subjects. Since the Clinical Test System is designed to operate only on earth, the table and gantry were not built to the size and mass requirements of a protoflight AMPDXA. In fact, the unit was built on a used CT Scanner structure. Employing used equipment for some of the structural elements and the rotating parts and machinery has allowed critical resources to be focused on the information extraction and analysis issues leading to human testing.

The image extraction capability of the AMPDXA is excellent. In a test using a human femur immersed in water, the BMD image is of higher resolution and the mass distribution in a projected thickness of a femur slice contains much more structural detail than conventional DXA's. The high frequency content of the BMD spatial projections are reproducible and provide information on the bone's microstructure. Work on multiple image analysis has progressed quite well. Using multiple projections about the bone axis allows structural properties (e.g., bending strength) to be obtained independent of patient position. To do this at least three arbitrary projections over 90 degrees (two of which are orthogonal) must be obtained. Such analysis can provide maximum and minimum moments of inertia for bending or torsion in any plane. Our

experiments to date with different sets of three projections show that the principal moments of inertia can be determined within 3 to 4%. Additional projections (above 3) reduce this number further. Our experimental systems also have some known non-linearities which when removed will drop the error in the three projection estimation of moments to less than 1 %.

Initial multiple projection work also shows that three projections are not sufficient for total image reconstruction; however, it appears that a cone beam type reconstruction from as few as three to seven projections is sufficient to produce a pseudo three-dimensional geometry that is mechanically equivalent to the measured hip.

The AMPDXA, as described above, has direct application to risk reduction in NASA's Critical Research Path. The AMPDXA is capable of real-time monitoring of bone and muscle loss at extremely high precision. Since the results are patient-specific and not tied to volumetric averages and statistical norms, the AMPDXA is a very useful tool for monitoring the effectiveness of countermeasures as well as determining the risk of fracture under deployment scenarios. The AMPDXA also appears to be a natural adjunct to earth-bound research on the effects of aging and disuse on bone integrity. It could also be used as a routine screening tool for osteoporosis and as a monitoring tool for osteoporosis drug therapy.

RESEARCH AREA:	**Technology Development**
PRINCIPAL INVESTIGATOR:	**Richard J. Cohen, M.D., Ph.D.**
ORGANIZATION:	**Massachusetts Institute of Technology**
PROJECT TITLE:	**Instrumentation for Non-Invasive Assessment of Cardiovascular Regulation**
FUNDING:	**$110,000 (FY 1998); $112,640 (FY 1999); $112,640 (FY 2000)**
TOTAL FUNDING:	**$335,280**

PROJECT EXECUTIVE SUMMARY

It is critically important to be able to assess alterations in cardiovascular regulation during and after space flight. We developed instrumentation for the non-invasive assessment of such alterations that can be used on the ground and potentially during space flight. This instrumentation will be used by the Cardiovascular Alterations Team at multiple sites for the study of the effects of space flight on the cardiovascular system and the evaluation of countermeasures. In particular, the Cardiovascular Alterations Team anticipates using this instrumentation in conjunction with ground-based human bed-rest studies and during application of acute stresses *e.g.*, tilt, lower body negative pressure, and exercise. In addition, the Cardiovascular Alterations Team anticipates using this instrumentation to study astronauts before and after space flight and ultimately, during space flight. The instrumentation may also be used by investigators in other physiologic areas related to space flight, such as neurovestibular, human performance, chronobiology, and psycho-social behavioral, to measure changes in autonomic nervous function.

The instrumentation is based on a powerful new technology – cardiovascular system identification (CSI) – which has been developed in our laboratory. CSI provides a non-invasive approach for the study of alterations in cardiovascular regulation. This approach involves the analysis of second-to-second fluctuations in physiologic signals such as heart rate and non-invasively measured arterial blood pressure in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of multiple physiologic mechanisms, CSI provides a closed-loop model of the cardiovascular regulatory state in an individual subject. Until now application of CSI currently required off-line computerized analysis of recorded physiologic signals by an expert user. The user interacted iteratively with the computer to preprocess the data, select data segments for analysis, run the CSI analyses, and evaluate and interpret the results. Thus the availability of this technology was limited to highly expert users located in Professor Cohen's laboratory. In this project, we developed integrated instrumentation capable of acquiring the physiologic signals, performing the CSI analysis in a fully automated fashion, and displaying the results on-line. The design of this instrumentation will be such that users with minimal training (including astronauts and other NSBRI investigators) can perform CSI onsite, conveniently and effectively. The availability of this instrumentation is essential for effectively studying the cardiovascular effects of space flight and for the subsequent development and evaluation of appropriate countermeasures. In particular this instrumentation will be used by the Cardiovascular Alterations Team in the study and development of countermeasures to the development of post-flight orthostatic hypotension. The development of such instrumentation may also have significant clinical impact on the diagnosis and treatment of patients with a variety of cardiovascular and neurological disorders.

RESEARCH AREA:	**Technology Development**
PRINCIPAL INVESTIGATOR:	**Richard S. Potember, Ph.D.**
ORGANIZATION:	**Johns Hopkins University Applied Physics Laboratory**
PROJECT TITLE:	**Miniature Time-of-Flight Mass Spectrometer**
FUNDING:	**$250,000 (FY 1998); $256,000 (FY 1999); $256,000 (FY 2000)**
TOTAL FUNDING:	**$762,000**

PROJECT EXECUTIVE SUMMARY

(1) Key Findings

Major advances must occur to protect astronauts from prolonged periods in near-zero gravity and high radiation associated with extended space travel. The dangers of living in space must be thoroughly understood and methods developed to reverse those effects that cannot be avoided. Six of the seven research teams established by the National Space Biomedical Research Institute (NSBRI) are studying biomedical factors for prolonged space travel to deliver effective countermeasures. To develop effective countermeasures, each of these teams require identification of and quantitation of complex pharmacological, hormonal, and growth factor compounds (biomarkers) in humans and in experimental animals to develop an in-depth knowledge of the physiological changes associated with space travel.

At present, identification of each biomarker requires a separate protocal. Many of these procedures are complicated and the identification of each biomarker requires a separate protocol and associated laboratory equipment. To carry all of this equipment and chemicals on a spacecraft would require a complex clinical laboratory, and it would occupy much of the astronaut's time. What is needed is a small, efficient, broadband medical diagnostic instrument to rapidly identify important biomarkers for human space exploration.

The Miniature Time-Of-Flight Mass Spectrometer Project in the Technology Development Team is developing a small, high resolution, time-of-flight mass spectrometer (TOFMS) to quantitatively measure biomarkers for human space exploration. Virtues of the JHU/APL TOFMS technologies reside in the promise for a small (less than one cubic ft), lightweight (less than 5 kg), low-power (less than 50 watts), rugged device that can be used continuously with advanced signal processing diagnostics. To date, we have demonstrated mass capability resolution from under 100 to beyond 10,000 atomic mass units (amu) in a very small, low-power prototype for biological analysis. Further, the electronic nature of the TOFMS output makes it ideal for rapid telemetry to earth for in-depth analysis by ground support teams.

A major objective of this project was the design and development of a mass spectrometer *system architecture* that can be utilized for diagnostics based on complex, non-volatile biomarkers species. Because this requires multiple (and generally incompatible) ionization sources, we have designed, built and tested an orthogonal extraction time-of-flight (TOF) mass spectrometer analyzer that incorporates a daul matrix-assisted laser desorption/ionization (MALDI) and electron ionization (EI) source.

The orthogonal extraction time-of-flight instrument was successfully completed and demonstrated. This novel instrument greatly expands the spectrum of biomarkers that can be measured by incorporating the capability of electric impact ionization with the previously demonstrated MALDI measurements. This new capability allows measurement of substances

ranging from low molecular volatile organic compounds to high molecular weight biological compounds such as proteins and carbohydrates.

The TOFMS Team has completed initial laboratory studies with critical biomarkers identified by the Muscle Alterations and Atrophy Team. The TOFMS Team has recorded full spectrum mass spectral signature of key target biomarker analytes using the MALDI technique at physiological concentrations found in urine. Sampling from urine has been chosen as a high priority for this project. Compounds investigated included: insulin-like growth factors (IGF-I), Urinary 3-methylhistidine, and estradiol. IGF-I is a potent anabolic factor that mimics most of the growth promoting actions of GH *in vivo.* IGF-1 has also been identified by the Bone Demineralization / Calcium Metabolism Team as an important biomarker.

Another biomarker identified by Muscle Alterations and Atrophy Team is urinary 3-methylhistidine. It is a measure of myofibrillar protein degradation. 3-methylhistidine cannot be re-utilized by the body. It is rapidly and quantitatively excreted in the urine. Estradiol is a steroid hormone important for the maintenance of muscle mass and bone density. It is widely speculated that steroid hormones such as estradiol play a central role in the early stages of muscle atrophy and bone demineralization.

The TOFMS team has also used matrix-assisted laser desorption mass spectrometry as a tool to *quantitatively* measure 3-MH in biological fluids. The TOFMS team analyzed various concentrations of 3-methylhistidine in water and in urine to determine the relationship between analyte concentration and analyte molecular ion intensity. The concentrations used in this study were based on 3-methylhistidine concentration typically found in urine, i.e. 20pmole -3.5nmole. The team examined the utility of two types of internal standards, histidine, a structural analogue, and d_3-3-methylhistidine, a stable-isotope labeled analogue. 3-Methylhistidine (3-MH) samples in water and urine were prepared ranging from 5uM-10mM, keeping the (3-MH)/(histidine) ratio constant at 1:10. Protonated molecular ions for 3MH and histidine could be identified; in the corresponding MALDI spectra. A plot of the ratio of relative peak intensities of (3MH)/(d_3-3-MH) versus 3-MH concentration gave a linear response with a correlation coefficient, R^2=0.9799 and a relative standard deviation of the slope of 4.00%.

The TOFMS Team has also completed initial laboratory studies with biomarkers specific to the Bone Demineralization / Calcium Metabolism Team. These include trivalent hydroxypyridinium crosslinks and creatinine. Trivalent hydroxypyridinium crosslinks are released into the circulation during bone resorption and are excreted as free pyridinolines molecules. In bone and cartilage, the collagen is bound by pyridinoline or deoxypyridinoline crosslinks. Deoxypyridinoline is found exclusively in bone while pyridinoline is found in skin, joint and cartilage. Creatinine is used to extrapolate the status of bone remodeling activity in various metabolic bone conditions.

The TOFMS Team has performed a mass spectral analysis of alendronate to determine the mass spectral pattern by MALDI and to add the compound to our library of critical biomarkers. Bisphosphonate administration to the hindlimb of suspended rats and limb immobilization studies in dogs suggest that this compound is an effective countermeasure to bone loss. Alendronate is a member of the bisphosphonate family of drugs used to treat/prevent osteoporosis. We analyzed a commercially available product, Fosamax.

The TOPMS team has also used a breath monitoring system to examine human subjects in order to select molecules that may serve as biomarkers of normal and abnormal physiology. These

molecules will be used to direct the selection of molecules to be monitored with the time-of-flight miniature mass spectrometer.

One of the objectives of the NSBRI Human Performance Factors, Sleep and Chronobiology Team is to develop strategies to monitor the circadian physiology of astronauts during long-duration space missions. The Team has identified that there is a critical need for in-flight assessment of melatonin levels. Melatonin is recognized as a very reliable marker of the human circadian pacemaker. Recent studies have indicated that there is very reliable correlation between the salivary and plasma levels. Because the sampling of plasma melatonin is an invasive procedure, it would be desirable to have a means of measuring salivary melatonin in subjects on long-duration space missions. We have performed a preliminary analysis of salivary melatonin using MALDI time-of-flight mass spectrometry of melatonin in saliva. Mass spectrometry may provide a reliable, convenient, and economical way to track melatonin during space missions.

Whole blood is the biological fluid of choice for therapeutic drug monitoring and for performing pharmacokinetic studies. Spectra for whole blood were recorded in DHB matrix and in cyano-4-hydroxycinnamic acid matrix. These spectra exhibited well-defined peaks from 100 to 400 mass units.

The risks to personnel in space from the naturally occurring radiation are generally considered to be one of the most serious limitations to human space missions. The NSBRI is examining the consequences of radiation in space *in vivo* in order to develop countermeasures, both physical and pharmaceutical, to reduce the risks of cancer and other diseases associated with such exposures. The consequences of exposure to radiation in space are considered a major limiting factor for long-duration interplanetary space travel for humans. Radiation doses in space may be hundreds of: times greater than those experienced on earth. These energetically charged particles can kill cells in the body or cause mutations that may lead to cancer, cataracts, central nervous system damage or other diseases.

The TOFMS Team evaluated three novel peptide cancer biomarkers to demonstrate the utility of MALDI-TOF as a tool for the early detection of carcinomas. The advantage in using it for detection over these other methods is the robust nature of the analyzer. MALDI-TOF mass spectrometry is rapid, sensitive, and tolerant of salts in biological samples.

(2) Summary of Satisfaction of Objectives

The first-year objectives were satisfactorily completed. In year one of the program the team prioritized the biological analytes that we would investigate. We recorded mass spectral signature data of initial biomarkers. We developed a quantification protocol to obtain biomarkers from blood, urine and breath. We coordinated TOFMS Development with other space experiments and devised a concept for a reflectron-TOFMS.

The second-year objectives were satisfactorily completed. The tasks accomplished in year 2 were to: build and test an electrospray sample deposition apparatus for quantitative analysis of biomarkers; evaluate and examine surfaces for sample inlet system; establish a quantitative method using isotopically labeled internal standards for specific biomarkers; develop a concept of operation for space-based processing of biomarkers from urine and blood; test the concept of operation on tabletop commercial TOFMS and DARPA "prototype" TOFMS; and, develop an orthogonal extration TOFMS design for enhanced ion collection efficiency.

The third-year objectives were satisfactorily completed. The tasks were to conduct subsystems integration; interface the system for interconnectivity and interoperability; determine TOFMS functionality; evaluate selected analytes provided by other collaborators on the TOFMS; and, evaluate analytical processing methods.

(3) Implications of this Project for Risk Reduction Related to the Critical Path

The long-term implications of this ground-based research and technology development project are to lay the scientific and engineering foundations to design, build and launch a flight-qualified "Miniature Time-of-Flight Mass Spectrometer" (TOFMS) for use on space platforms such as the Space Shuttle and the International Space Station (ISS). Successful deployment of this instrument in near-earth missions will lay medial, engineering and scientific groundwork to adopt this medical diagnostic instrument for a mission to Mars later this century.

The development of the "Miniature Time-of-Flight Mass Spectrometer" will provide NSBRI/NASA with a complete medical diagnostic system to measure bone, internal organs, and soft tissues, routinely and non-invasively. This compact medical diagnostic system will provide autonomous and semi-autonomous patient monitoring systems with low false positive alarm rates.

This research project supports the goals of the Life Sciences Division of NASA to aid in the exploration of the solar system, support the achievement of routine space travel, and enrich life on Earth through the use of space technology and the application of biomedical knowledge. This research project falls into Category 4: Clinical Research in Support of Space Missions (Medicine in Extreme Environments).

The Countermeasure Readiness Level (CRL) developed by NASA describes the level of scientific maturity of applied research from the development of a hypothesis to validated procedure ready for operational implementation of procedures and devices. This scale has been developed as a method to mitigate the deleterious effects on humans engaged in space flight. Using this scale as a metric, this project was at level 5, "Proof of concept testing and initial demonstration of feasibility and efficacy." Based on the results that we have achieved to date, we believe that this project can successfully transition to countermeasurement development.

RESEARCH AREA:	**Technology Development**
PRINCIPAL INVESTIGATOR:	**Richard H. Maurer, Ph.D.**
ORGANIZATION:	**Johns Hopkins University Applied Physics Laboratory**
PROJECT TITLE:	**In-Situ Spectrometry of Neutrons**
FUNDING:	**$280,000 (FY 1998); $316,720 (FY 1999); $286,720 (FY 2000)**
TOTAL FUNDING:	**$883,440**

PROJECT EXECUTIVE SUMMARY

High-energy charged particles of extra-galactic, galactic and solar origin collide with spacecraft structures in Earth orbit outside the atmosphere and in interplanetary travel beyond the Earth's magnetosphere. These primaries create a number of secondary particles inside the structures that can produce a significant ionizing radiation environment. This radiation is a threat to long term inhabitants or travelers for space missions and produces an increased risk of cancer and DNA damage. The primary high energy cosmic rays and trapped protons collide with common spacecraft materials such as aluminum and silicon and create secondary particles inside structures that are mostly protons and neutrons. Indeed, the effect of tens of grams per square centimeter of structure or atmosphere is to convert and multiply the primary proton "beam" into a secondary environment dominated by neutrons between several MeV and several tens of MeV. Charged protons are readily detected and instruments are already in existence for this task. Neutrons are electrically neutral and therefore much more difficult to measure and detect. These neutrons are reported to contribute 30-60% of the dose inside space structures and cannot be ignored. Currently there is no compact, portable and real time neutron detector instrumentation available for use inside spacecraft or on planetary surfaces where astronauts will live and work.

Present neutron detection systems use gas tube proportional counters for the monitoring of low energy (0.025 eV to 1 MeV) neutrons. However for higher energies the detector systems are quite large and massive and often employ passive detection methods that must be recycled and read out after the fact. Physically large neutron diffraction tables are used for accelerator experiments. Emulsions are flown on the Space Shuttle and returned to Earth for analysis. The NASA Ames aircraft uses an instrument built with Bonner spheres which are large spheres of polyethylene moderator some tens of centimeters in diameter with a photodiode in the center and weighing 1500 pounds.

In 1997 we proposed to design and build a portable, low power and robust neutron spectrometer that measures the neutron spectrum from 10 KeV to 500 MeV with at least 10% energy resolution in the various energy intervals. This instrument will monitor the existing neutron environment both inside spacecraft structures and on planetary surfaces to determine the safest living areas, warn of high fluxes associated with solar storms and assist the NSBRI Radiation Effects Team in making an accurate assessment of increased cancer risk, DNA and central nervous system (CNS) damage to astronauts. The instrument uses a highly efficient proportional counter Helium 3 tube at the lowest energy intervals where equivalent damage factors for tissue are the highest (10 KeV-2 MeV). The Helium 3 tube is shielded with a cadmium absorber to eliminate the much less damaging and, hence, uninteresting, but more prevalent, thermal and epithermal neutrons and to make the structure of the spectrum more accurate in the 20 KeV-2 MeV range. A second option is to use a pair of tubes, one shielded and one unshielded, combining the difference in their counts to yield the thermal neutron contribution. The spectrometer also uses a 5mm lithium drifted bulk silicon solid state detector in the neutron

energy range of 2-500 MeV due to its demonstrated and modeled detection efficiency of 3-5% in the 5-150 MeV energy range. In high energy regions equivalent damage factors for dose equivalent are lower but hits from one or a small number of neutrons may prove to be important in sensitive localized volumes. The silicon detector system for high energy neutrons will discriminate against charged particles by using a plastic cesium iodide scintillator of an appropriate geometry (a small cup and plug configuration for a Mars Lander; a surrounding rectangular liner for a Space Station Express Rack) monitored by a silicon PIN photodiodes.

The first round of experiments with monoenergetic neutron beams on the Helium 3 tube and 5mm silicon detector systems were performed in February and June 1999. Both detector systems have previously been evaluated with Californium (mean energy ~ 1 MeV) and Americium/Beryllium (mean energy ~5 MeV) radioactive neutron sources at NIST. The Helium 3 tube exhibited energy resolution of at least 1 KeV over the energy range from 10 KeV to 3 MeV. The efficiency of detection of the tube decreased from 80% at energies of tens to hundreds of KeV to 0.1% as 1 MeV was approached as would be expected from the behavior of the neutron capture cross section for the neutron-Helium 3 reaction over this energy range. The best performing high-energy silicon detector from the set of FY 1999 tests proved to be the 5mm thick lithium drifted silicon detector. It demonstrated surprisingly good efficiencies of 1-5% at the Columbia RARAF (Radiological Research Accelerator Facility) for neutron beam energies of 2.46, 5.89 and 14, 16.25 and 18.5 MeV.

The 5 millimeter thickness of the silicon detector is a reasonable fraction of the neutrons' mean free paths in silicon in the 2-150 MeV energy range thereby significantly increasing the probability of a neutron-silicon nucleus interaction. We plan to overlap the detection of neutrons with the Helium 3 tube in the 2-5 MeV energy interval for cross calibration purposes. In the energy region above 20 MeV the substantial progress that we have made with modeling and experiments in FY 1999 and 2000 has yielded a conceptual design of one or more thick bulk lithium drifted silicon detectors (5-7 mm in an array or ganged together to increase count rate or roughly double the detection efficiency respectively) surrounded by an appropriately configured charged particle anti-coincidence scintillator detection system. Results from simulations of any high-energy stack or proton recoil telescope configuration in January and March 1999 showed that only detection efficiencies of 5X10 -4 would be achieved while maintaining 10% energy resolution for neutrons of energy greater than 50 MeV. Results from the RARAF experiments in February and June 1999 showed that the 5mm bulk silicon detector had as much as 5% (5X10 -2) efficiency between 10 and 20 MeV. Study of the Evaluated Nuclear Data Files (ENDF) archived by the Department of Energy showed that the total cross section for the neutron-silicon reaction remains fairly constant up to 150 MeV; thus, the bulk silicon detector efficiency is expected to do the same. The two order of magnitude advantage in detection efficiency for the bulk detector versus the recoil telescope disqualified the latter.

The late summer and fall of FY 1999 were occupied with two main activities: 1) our proposal to NASA in response to AO 99-HEDS-01 for a neutron spectrometer on the Mars 2003 Lander; and 2) the design and purchase of a set of detectors and associated electronics including battery packs for an engineering prototype instrument to be qualified for aircraft flight. NASA/Dryden committed to several F 15/18 flights in January 1999 for our spectrometer to monitor avionics environment neutrons and help NASA check out our design.

The proposal for MANES (MArtian Neutron Energy Spectrometer) was submitted in August 1999. It was selected for a stage of further definition in November 1999 as a potential instrument for the then-scheduled Mars 2003 Lander. The status of this instrument and its funding has

evolved from potential individual Mars 2003 Lander instrument (December 1999-February 2000), to possible combination with the JSC MARIE instrument after cancellation of the 2001 Lander in April (March-May 2000), to proposal for an extended definition phase for a 2005 Lander instrument after cancellation of the large 2003 Lander and selection of the two-Athena-Rovers-in-a-bag for 2003 in July 2000 (May-August 2000), to being on hold pending a decision to fly a large Lander in 2005. That next decision on the Mars Surveyor program is expected in late September/October 2000. The changing situation has required much communication, alertness, revised statements of work and costing in addition to the original proposal with both NASA Headquarters and Johnson Space Center (JSC). A grant of $123,000 was received from JSC for accommodation and definition phase work on MANES from February to August of 2000.

The efforts on the aircraft flight hardware were interrupted by the MANES proposal. Fabrication occurred in September-October 1999. Included in this two-month period were rise time discrimination experiments with the Helium3 tube detector at RARAF at the end of October. Pictures of the instrument fabricated for aircraft flight are included in Appendix A of this report and represent the engineering prototype hardware proposed and promised to NSBRI in 1997. The instrument actually exceeds the original expectations in that it has been packaged and qualified for an uncontrolled aeronautics environment. It is more than just a laboratory instrument.

The aircraft instrument was ready for qualification in November 1999 when our selection for the Mars 2003 Lander interrupted its progress again. Analysis of its mechanical integrity for flight vibration and acoustics did continue in the November 1999-January 2000 time frame. The results of the analysis required some strengthening of the instrument and an additional cover for acoustic dampening of the Helium3 tube. The actual vibration and temperature/altitude qualification test finally took place in February 2000. The instrument readily passed the vibration test and operated successfully at low temperature; however, an isolated corona breakdown occurred in one of the high voltage supply systems at a simulated altitude of 45,000 feet. The point of voltage break down was found and fixed and the instrument was successfully qualified to the simulated 45,000 feet. The instrument was delivered to NASA Dryden on March 23, 2000 and flown the week of April 24-28, 2000 on an F15 aircraft.

We took data up to 39,000 feet on ascent (the plane was to fly at a planned 40,000 feet cruise level) when we experienced a corona breakdown in the high voltage supply systems for both detectors. We are implementing and will implement more comprehensive and robust fixes for each high voltage supply system and plan to repeat the aircraft flights when new funding is received in FY 2001.

In January 2001 we were notified that our proposal titled "Development of a Neutron Spectrometer to Assess Biological Radiation Damage Behind Spacecraft Materials" submitted in March 1999 in response to NASA NRA 98-Heds-05 would be funded for a period of 3.5 years from May 2000 to November 2003 at a level of $90,000 per year for a total of $315,000. Our primary responsibility under this grant is to support Lawrence Berkeley Laboratory (LBL) personnel in the evaluation of spacecraft structural and shielding materials by supplying a version of the neutron spectrometer compatible with ground-based accelerator research. Due to the unavailability of accelerator facilities our first scheduled test date is now January 2001.

The major effort in detector evaluation that took place in 2000 was a series of experiments at the Los Alamos Neutron Science Center (LANSCE) to measure energy deposition in the 5mm thick

lithium drifted silicon detector by neutrons with an energy range from 20-600 MeV. The experiments were performed by integrating our 5mm silicon detector with the LANSCE time-of-flight neutron spectrometer on the 90 meter beam line to give simultaneous measurements of the incident neutron energy (LANSCE fission chamber) and energy deposited in our detector. Energy depositions of up to 150 MeV were seen from the up to 600 MeV incident neutrons in our 5mm detector. Complete analysis of the data from these experiments is on hold pending the resumption of funding in FY 2001. These high-energy neutron experiments complement and complete the measurement of neutron-silicon energy depositions from known monoenergetic neutrons begun at RARAF in1999. Together the LANSCE and RARAF neutron exposure data will enable us to develop a complete response function for the 5mm detector between neutron energies of 2 and 600 MeV. Inversion of this response function will allow us to calculate a most likely incident neutron energy spectrum, previously unknown, from a measured energy deposition spectrum.

The modeling component of this research program occurred on a continuous basis in FY 2000 until funds expired. We concentrated on modeling the high-energy channel from detailed cross sections of the basic neutron-silicon interactions using state-of-the-art computer codes. There are four reasons to develop this advanced modeling capability: 1) to assess the accuracy of the codes themselves to predict energy deposition in a silicon detector (by comparison with experimental data); 2) to use the codes in understanding the experimental results; 3) to determine whether the codes can be used to calculate the shielding and scattering effects of the instrument packaging and surrounding environment (structure or atmosphere); 4) to assess the ability of the codes to supplement the determination of the instrument response function at interpolated and extrapolated energies (since it is impractical to test at intervals of 10 MeV for the whole energy range). We have found that the GEANT4 code originally developed at CERN is the easiest to use, is maintained in a timely fashion by its developers and reproduces our RARAF (2-20 MeV) energy deposition data reasonably well.

NSBRI RESEARCH PROGRAM
SYNERGY PROJECTS

Cohen, R. J.	**PI**	**MIT**	**Alterations in Cardiovascular Regulation and Function During Long-Term Simulated Microgravity (Cardiovascular Alterations – Bone Loss)**	**130**
LeBlanc, A.	CO-I	Baylor		
Ramsdell, C. D.	CO-I	Harvard		
Shackelford, L.	CO-I	NASA JSC		
Yelle, J. M.	CO-I	NASA JSC		
Dijk, D.	**PI**	**Harvard**	**Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and the Renin-Angiotensin System (Cardiovascular Alterations – Human Performance)**	**132**
Cajochen, C.	CO-I	Harvard		
Williams, G. H.	CO-I	Harvard		
Ramsdell, C. D.	**PI**	**Harvard**	**Visual- and Vestibular-Autonomic Influence on Short-Term Cardiovascular Regulatory Mechanisms (Cardiovascular Alterations – Neurovestibular Adaptation)**	**134**
Beall, A. C.	CO-I	MIT		
Mullen, T. J.	CO-I	Harvard		
Oman, C. M.	CO-I	MIT		
Paloski, W.	CO-I	NASA JSC		
Wood, S.	CO-I	Baylor		
Yelle, J. M.	CO-I	NASA JSC		
Mullington, J. M.	**PI**	**Harvard**	**Sustained Partial Sleep Deprivation: Effects on Immune Modulation & Growth Factors (Human Performance – Immunology, Infection & Hematology – Muscle Alterations)**	**136**
Butel, J. S.	CO-I	Baylor		
Dinges, D. F.	CO-I	Penn		
Lednicky, J. A.	CO-I	Baylor		
Ling, P. D.	CO-I	Baylor		
Schwartz, R. J.	CO-I	Baylor		

SYNERGY AREA:	**Cardiovascular Alterations - Bone Loss**
PRINCIPAL INVESTIGATOR:	**Richard J. Cohen, M.D., Ph.D.**
ORGANIZATION:	**Harvard Medical School/MIT**
PROJECT TITLE:	**Alterations in Cardiovascular Regulation and Function During Long-Term Simulated Microgravity**
FUNDING:	**$24,968 (FY 1998); $24,969 (FY 1999)**
TOTAL FUNDING:	**$49,937**

PROJECT EXECUTIVE SUMMARY

The Cardiovascular Alterations Team is conducting studies of hemodynamic regulation and susceptibility to arrhythmias resulting from sixteen days of simulated microgravity exposure. In these studies very intensive measurements are made during a short duration of bed rest. In this collaborative effort are making many of the same measurements, however much less frequently, on subjects who are exposed to a much longer duration of simulated microgravity.

Alterations in cardiovascular regulation and function that occur during and after space flight have been reported. These alterations are manifested, for example, by reduced orthostatic tolerance upon reentry to the earth's gravity from space. However, the precise physiologic mechanisms responsible for these alterations remain to be fully elucidated. Perhaps, as a result, effective countermeasures have yet to be developed. In addition, numerous reports from the past 30 years suggest that the incidence of ventricular arrhythmias among astronauts is increased during space flight. However, the effects of space flight and the associated physiologic stresses on cardiac conduction processes are not known, and an increase in cardiac susceptibility to arrhythmias has never been quantified.

In this project we are applying the most powerful technologies available to determine, in a ground-based study of long duration space flight, the mechanisms by which space flight affects cardiovascular function, and then on the basis of an understanding of these mechanisms to develop rational and specific countermeasures. To this end we are conducting a collaborative project with the Bone Demineralization/Calcium Metabolism Team of the National Space Biomedical Research Institute (NSBRI). The Bone Team is conducting bed rest studies in human subjects lasting 17 weeks, which provides a unique opportunity to study the effects of long duration microgravity exposure on the human cardiovascular system. We are applying a number of powerful new methods to these long term bed rest subjects, including cardiovascular system identification (CSI), microvolt level T wave alternans analysis, and cardiac magnetic resonance imaging to assess non-invasively the effects of simulated long duration space flight on the cardiovascular system.

CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate, arterial blood pressure (ABP), and instantaneous lung volume (ILV – respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of all the physiologic mechanisms coupling these signals, CSI provides a model of the closed-loop cardiovascular regulatory state in an individual subject. The model includes quantitative descriptions of the heart rate baroreflex as well as other important physiologic mechanisms. With an additional non-invasive measurement of stroke volume (SV – ultrasound Doppler method), the model may be extended to also include the characterization of the peripheral

resistance baroreflex – which may play a central role in the development of orthostatic intolerance – and measures of systolic and diastolic function.

To determine whether simulated long-term space flight increases the risk of developing life-threatening heart rhythm disturbances such as sustained ventricular tachycardia (defined as ventricular tachycardia lasting at least 30 seconds or resulting in hemodynamic collapse) and ventricular fibrillation, we are applying a powerful new non-invasive technology, developed in Professor Cohen's laboratory at MIT, for the quantitative assessment of the risk of life-threatening ventricular arrhythmias. This technology involves the measurement of microvolt levels of T wave alternans during exercise stress. In addition, we are obtaining 24-hour Holter monitoring to detect non-sustained ventricular tachycardia and to assess heart rate variability. Finally, in order to investigate the effect of long duration microgravity on cardiac mass, cardiac magnetic resonance images are being obtained before and after the bed rest period.

To date, measurements for CSI, 24-hour Holter monitoring, and cardiac magnetic resonance imaging have been made on seven long-term bed rest subjects. Measurements for TWA analysis have been made in four of these subjects. The studies are still ongoing, and only preliminary analysis of the data has been completed. During Year 3 we will complete the analysis of the data generated in this study.

SYNERGY AREA:	**Human Performance - Cardiovascular Alterations**
PRINCIPAL INVESTIGATOR:	**Derk-Jan Dijk, Ph.D.**
ORGANIZATION:	**Harvard Medical School**
PROJECT TITLE:	**Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and Renin-Angiotensin System**
FUNDING:	**$24,896 (FY 1998); $24,897 (FY 1999)**
TOTAL FUNDING:	**$49,793**

PROJECT EXECUTIVE SUMMARY

Total sleep deprivation leads to decrements in neurobehavioral performance and changes in electroencephalographic (EEG) oscillations as well as the incidence of slow eye movements ad detected in the electro-oculogram (EOG) during wakefulness. Although total sleep deprivation is a powerful tool to investigate the association of EEG/EOG and neurobehavioral decrements, sleep loss during space flight is usual only partial. Furthermore exposure to the microgravity environment leads to changes changes in sodium and volume homeostasis and associated renal and cardio-endocrine responses. Some of these changes can be induced in head down tilt bedrst studies. We integrate research tools and research projects to enhance the fidelity of the simulated conditions of space flight which are characterized by complexity and mutual interactions. The effectiveness of countermeasures and physiologic mechanisms underlying neurobehavioral changes and renal-cardio endocrine changes are investigated in Project 3 of the Human Performance Team and Project 3 of the Cardiovascular Alterations Team respectively. Although the specific aims of these two projects are very different, they employ very similar research protocols. Thus, both projects investigate the effects of posture/bedrest and sleep deprivation (total or partial) on outcome measures relevant to their specific aims. The main aim of this enhancement grant is to exploit the similarities in research protocols by including the assessment of outcome variables relevant to the Renal-Cardio project in the research protocol of Project 3 of the Human Performance Team and by including the assessment of outcome variables relevant to the Quantitative EEG and Sleep Deprivation Project in the research protocols of Project 3 of the Cardiovascular Alterations team. In particular we will assess Neurobehavioral Function and Waking EEG in the research protocols of the renal-cardio endocrine project and renin-angiotensin and cardiac function in the research protocol of the Quantitative EEG and Waking Neurobehavioral Function project. This will allow us to investigate two additional specific aims:

1) Test the hypothesis that chronic partial sleep deprivation during a 17 day bed rest experiment results in deterioration of neurobehavioral function during waking and increases in EEG power density in the theta frequencies, especially in frontal areas of the brain, as well as the nonREM-REM cycle dependent modulation of heart-rate variability.

2) Test the hypothesis that acute total sleep deprivation modifies the circadian rhythm of the renin-angiotensin system, changes the acute responsiveness of this system to posture beyond what a microgravity environment alone does and affects the nonREM-REM cycle dependent modulation of heart-rate variability.

The data obtained on the waking EEG and neurobehavioral function in the chronic partial sleep deprivation experiment will complement the data obtained on the effects of total sleep deprivation which are collected in project 3 of the Human Performance Team. The data obtained

on the renin-angiotensin levels in the acute total sleep deprivation experiment will complement data obtained on the effects of chronic partial sleep deprivation which will be collected in project 3 of the Cardiovascular Alterations team. We have obtained recording in two subjects who participated in a 24 day laboratory study with 21 days of continuous bedrest. The application of identical research tools and outcome measures in research protocols across the Cardiovascular and Human Performance team will greatly enhance the overall science return of these projects and emphasizes the synergistic nature of this application.

SYNERGY AREA:	**Cardiovascular Alterations - Neurovestibular Adaptation**
PRINCIPAL INVESTIGATOR:	**Thomas J. Mullen, Ph.D. (1998)** **Craig D. Ramsdell, M.D. (1999)**
ORGANIZATION:	**Harvard Medical School**
PROJECT TITLE:	**Visual- and Vestibular-Autonomic Influence on Short-term Cardiovascular Regulatory Mechanisms**
FUNDING:	**$24,983 (FY 1998); $24,985 (FY 1999)**
TOTAL FUNDING:	**$49,968**

PROJECT EXECUTIVE SUMMARY

This synergy project was a one-year effort conducted cooperatively by members of the NSBRI Cardiovascular Alterations and Neurovestibular Adaptation Teams in collaboration with NASA-Johnson Space Center (JSC) colleagues. The objective of this study was to evaluate visual-autonomic interactions on short-term cardiovascular regulatory mechanisms. Based on established visual-vestibular and vestibular-autonomic shared neural pathways, we hypothesized that visually induced changes in orientation will trigger autonomic cardiovascular reflexes. A second objective was to compare baroreflex changes during postural changes as measured with the new Cardiovascular System Identification (CSI) technique with those measured using a neck barocuff. While the neck barocuff stimulates only the carotid baroreceptors, CSI provides a measure of overall baroreflex responsiveness.

This study involved a repeated measures design with 16 healthy human subjects (8 M, 8 F) to examine cardiovascular regulatory responses during actual and virtual head-upright tilts. Baroreflex sensitivity was first evaluated with subjects in supine and upright positions during actual tilt-table testing using both neck barocuff and CSI methods. The responses to actual tilts during this first session were then compared to responses during visually induced tilt and/or rotation obtained during a second session.

Effect of actual changes in posture on baroreflex responses. CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate (HR), arterial blood pressure (ABP), and instantaneous lung volume (ILV, respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. A random interval breathing protocol (mean rate of 12 breaths per minute, inter-breath intervals randomly varying between one and 15 seconds) is utilized to broaden the frequency content of the recorded physiological signals, thereby facilitating CSI. Using the CSI technique, we have previously observed significant alterations to the autonomically mediated coupling mechanisms with a change in posture from supine to upright, while non-autonomically mediated mechanisms are left essentially unchanged. Further analysis of data from this first session will utilize CSI measurements to confirm this result, and to quantitatively compare the neck barocuff method with CSI in estimating baroreflex sensitivity.

Carotid baroreflex responses were obtained in both supine and head upright tilt positions using the neck barocuff employed according to the method described by Fritsch *et al.* (1992). This technique allows assessment of vagally mediated carotid baroreceptor-cardiac reflex responses provoked by neck pressure and suction steps during held expiration. Pressure was increased to 40 mmHg for 5 cardiac cycles, reduced by 15 mmHg decrements after each of the next seven R waves to -65 mmHg, and finally returned to ambient levels. Responses from up to four

successful repetitions of this stimulus sequence during both supine and upright positions were averaged. R-R intervals were plotted against carotid distending pressure (taken to be systolic minus neck chamber pressures). There were significant differences between male and female subjects for minimum, maximum and control RR interval ($p<0.01$). For both male and female subjects, there were highly significant decreases ($p<0.0001$) in minimum, maximum and control RR intervals when subjects were tilted from the supine to upright position. There were not; however, significant differences in either the RR interval ranges or maximum slopes between these positions.

Cardiovascular responses during virtual tilt and/or rotation. A second session with the same subjects was then used to examine the effects of visually induced virtual tilt and/or rotation stimuli in modulating autonomic cardiovascular reflexes. One of the stimuli involved a simple "mirror bed" to provide an illusion of body tilt without rotation. This device involved mounting a mirror over a subject in a supine orientation to align surrounding visual vertical cues with the subject's longitudinal body axis. In addition to the mirror bed, visually induced tilt and/or rotation illusions were elicited by a full-field virtual environment generator at NASA known as the Preflight Adaptation Trainer DOME. The subject was supine with the head positioned near the center of this large spherical DOME. A virtual scene aligned with the longitudinal body axis was then rotated in the subject's pitch, yaw or roll planes to elicit sensations of tilt and/or rotation. The pitch and yaw DOME visual stimuli rotated about an earth horizontal axis producing the paradoxical sense of tilt and rotation. The roll visual stimulus, on the other hand, rotated about an earth vertical axis typically resulting in the sense of rotation without tilt.

The visual conditions were therefore chosen to provide the following combinations of perceived tilt and/or rotation:

Mirror bed – perceived tilt without rotation
DOME Pitch and Yaw – perceived tilt and rotation
DOME Roll – perceived rotation without tilt

Although there was a high degree of variability across subjects, the mean responses reflect the expected combinations of perceived tilt and rotation described above. The mirror bed was rated by subjects to be the most compelling, with the perceived orientation of the head (54.7 ± 6.7, mean $\pm$SEM) slightly greater than the perceived orientation of the body (45.0 ± 5.7). Cardiovascular responses were recorded during 2 min prior to the start of each virtual tilt and during the initial three minutes with eyes open. Although the data appear to be quite variable, there were a few instances when the changes were quite dramatic. For example, rapid decreases in both systolic and diastolic pressure were observed in some subjects at the onset of the virtual tilt similar to the changes in blood pressure to an actual change in body posture on a tilt table.

Our preliminary results suggest that visually induced virtual tilt can elicit at least transient cardiovascular changes in some individuals. Pending further analysis, we expect to find that the degree of change in cardiovascular reflexes will correlate with individual measures of tilt perception. We will further characterize these effects on cardiovascular regulatory mechanisms using CSI, and expect that visually induced tilts will result in reductions in HR baroreflex sensitivity. The significance of these findings is that virtual environment stimuli may be used in the future to enhance cardiovascular and/or vestibular countermeasures for long-duration spaceflight.

SYNERGY AREA:	**Human Performance - Immunology, Infection and Hematology - Muscle Alterations**
PRINCIPAL INVESTIGATOR:	**Janet M. Mullington, Ph.D.**
ORGANIZATION:	**Harvard Medical School**
PROJECT TITLE:	**Sustained Partial Sleep Deprivation: Effects on Immune Modulation & Growth Factors**
FUNDING:	**$25,000 (FY 1998); $25,000 (FY 1999)**
TOTAL FUNDING:	**$50,000**

PROJECT EXECUTIVE SUMMARY

The vulnerability to medical emergencies is greatest in space where there are real limits to the availability or effectiveness of ground based assistance. Moreover, astronaut safety and health maintenance will be of increasing importance as we venture out into space for extended periods of time. It is therefore critical to understand the mechanisms of the regulatory physiology of homeostatic systems (sleep, circadian, neuroendocrine, fluid and nutritional balance) and the key roles played in adaptation. This synergy project has combined aims of the *"Human Performance Factors, Sleep and Chronobiology Team"*; the *"Immunology, Infection and Hematology Team"*; and the *"Muscle Alterations and Atrophy Team"*, to broadly address the effects of long term sleep reduction, as is frequently encountered in space exploration, on neuroendocrine, neuroimmune and circulating growth factors. Astronaut sleep is frequently curtailed to averages of between 4-6.5 hours per night. There is evidence that this amount of sleep is inadequate for maintaining optimal daytime functioning. However, there is a lack of information concerning the effects of chronic sleep restriction, or reduction, on regulatory physiology in general, and there have been no controlled studies of the cumulative effects of chronic sleep reduction on neuroendocrine and neuroimmune parameters.

This synergy project represents a pilot study designed to characterize the effects of chronic partial sleep deprivation (PSD) on neuroendocrine, neuroimmune and growth factors. This project draws its subjects from two (of 18) conditions of the larger NSBRI project, *"Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight" (PI:David Dinges)*, one of the projects on the *"Human Performance Factors, Sleep and Chronobiology Team"*. For the purposes of this study, to investigate the effects of chronic sleep loss on neuroendocrine and neuroimmune function, we have focused on the two extreme sleep conditions from this larger study: a 4.2 hour per night condition, and a 8.2 hour per night condition.

During space flight, muscle mass and bone density are reduced, apparently due to loss of GH and IGF-I, associated with microgravity. Since >70% of growth hormone (GH) is secreted at night in normal adults, we hypothesized that the chronic sleep restriction to 4 hours per night would reduce GH levels as measured in the periphery. In this synergy project, in collaboration with the *"Muscle Alterations and Atrophy Team"*, we have measured insulin-like growth factor-I (IGF-I) in peripheral circulation to test the prediction that it will be reduced by chronic sleep restriction.

In addition to stress, recent research suggests that sleep is also involved in modulation of immune function. While we all have the common experience of being sleepy when suffering from infection, and being susceptible to infection when not getting enough sleep, the mechanisms involved in this process are not understood and until recently have gone largely overlooked. We

believe that the immune function changes seen in spaceflight may also be related to the cumulative effects of sleep loss. Moreover, in space flight, the possibility of compromised immune function or of the reactivation of latent viruses are serious potential hazards for the success of long-term missions. Confined living conditions, reduced sleep, altered diet and stress are all factors that may compromise immune function, thereby increasing the risks of developing and transmitting disease. Medical complications, which would not pose serious problems on earth, may be disastrous if they emerged in space. Understanding the long-term consequences of sleep curtailment on general health and physiological functioning is critical to the success of any space mission where astronauts will be away from critical care facilities for extended periods of time.

NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH

Merfeld, D.	**PI**	**Harvard**	**Decoding of Graviceptor Cues, Including Adaptive Changes**	**139**
Wall, C. C.	CO-I	Harvard		
Zupan, L.	CO-I	Harvard		
Peterka, R.	CO-I	Oregon Health Sci.		
Shelhamer, M. J.	CO-I	Hopkins/SOM		
Seidman, S.	**PI**	**Rochester**	**Plasticity in the Vestibuloocular Reflexes and Perception**	**140**
Paige, G.	CO-I	Rochester		
Angelaki, D.	**PI**	**Washington University**	**Neural Mechanisms of Vestibular Adaptation**	**141**
Dickman, J. D.	CO-I	U of Miss.		
King, W. M.	**PI**	**U of Miss.**	**Signal Processing and Adaptation in Central Otolith Pathways**	**142**
Zhou, W.	CO-I	U of Miss.		
Raymond, J.	**PI**	**Stanford**	**Vestibular and Visual Control of Eye Movement**	**143**
Cohen, H. S.	**PI**	**Baylor**	**Management of Adaptation to Altered Sensorimotor States**	**144**
Bloomberg, J. J.	CO-I	NASA JSC		

RESEARCH AREA:	**NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH**
PRINCIPAL INVESTIGATOR:	**Daniel Merfeld, Ph.D.**
ORGANIZATION:	**Harvard Medical School**
PROJECT TITLE:	**Decoding of Graviceptor Cues, Including Adaptive Changes**
FUNDING:	**$294,427 (FY 1999); $259,829 (FY 2000)**

PROJECT EXECUTIVE SUMMARY

The long term goal of this research is to understand how sensory cues from the semicircular canals (measuring rotation) and the visual system (measuring orientation and motion) influence the interpretation of sensory cues from the otolith organs (measuring gravity and linear acceleration). To accomplish this goal, this proposal focuses on the study of adaptive changes in the interpretation of otolith cues caused by transitions to altered gravitational environments. Understanding these key systems-level processes underlying vestibular compensation and dynamic adaptation will provide the foundation to facilitate the development of targeted behavioral approaches for the management of balance and vestibular disorders and adaptation to altered gravitational environments. The seven specific aims of this grant are: 1. Investigate how the human nervous system resolves otolith measurements of gravito-inertial force into estimates of gravity and linear acceleration in a 1-G environment. 2. Investigate how the nervous system resolves gravito-inertial force into estimates of gravity and linear acceleration in hypergravic environments. 3. Investigate adaptive changes in how the human nervous system resolves otolith measurement of gravito-inertial force into estimates of gravity and linear acceleration in hypergravic environments. 4. Investigate to what extent responses to high frequency inertial stimuli, normally interpreted primarily as translation, can be adapted to yield increased tilt responses. 5. Investigate to what extent responses to low frequency inertial stimuli, normally interpreted primarily as tilt, can be adapted to yield increased translation responses. 6. Investigate to what extent context specific adaptation of graviceptor mediated tilt and translation occurs. 7. Develop and test our systems approach model of visual-vestibular interactions. The experimental specific aims will be addressed by measuring eye movements and perceptual responses. The modeling specific aim will be addressed using numerical computer simulations.

RESEARCH AREA:	**NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH**
PRINCIPAL INVESTIGATOR:	**Scott Seidman, Ph.D.**
ORGANIZATION:	**University of Rochester**
PROJECT TITLE:	**Plasticity in the Vestibuloocular Reflexes and Perception**
FUNDING:	**$234,161 (FY 1999); $239,507 (FY 2000)**

PROJECT EXECUTIVE SUMMARY

The vestibular system serves to maintain orientational, postural, and visual stability during head movements. The vestibulo ocular reflex (VOR) is an important vestibular mechanism that rotates the eyes to maintain binocular fixation on a target during head movement, thus maintaining clear and stable vision. The VOR is actually a set of reflexes activated by two forms of head acceleration; angular (the AVOR), driven by the semicircular canals, or linear (the LVOR), driven by the otoliths. Th otolith organs sense linear accelerations caused by both head translation and head tilt relative to gravity, yet each form of motion requires different compensatory responses. Thus, The LVOR is comprised of both translational and tilt forms. The vestibular system may undergo functional changes in response to development, aging, or disease processes, but is able to modify its behavior such that binocular fixation is maintained during head movements. This process, known as adaptive plasticity, is well recognized in the AVOR, but is largely unstudied in the LVOR. We will study adaptive plasticity of the AVOR and translational LVOR in response to novel visual/vestibular challenges. The resulting behavior of these challenges on the AVOR and LVOR will be assessed and compared. This research will elucidate the extent to which the AVOR, translational LVOR, and tilt LVOR, are driven by shared neural pathways, or if control of each subsystem is independent. Selective adaptation of the VOR will be exploited to formulate structural and functional characteristics of central VOR pathways, and suggest stages of neuronal processing where adaptation may occur. In addition, VOR and motion perception mechanisms share common sensory inputs, as well as the common goal of maintaining spatial orientation. To determine if VOR and motion perception share common pathways, the effects of AVOR and translational LVOR adaptation on the perception of motion and tilt will also be examined. The study of LVOR adaptation will directly contribute to the understanding of how the vestibular system adapts to natural development, disease and aging processes, and to the unnatural gravitational environments encountered during air and space travel. A better understanding of LVOR adaptation will catalyze test of susceptibility of motion sickness (including the form experienced in space flight), and countermeasures to prevent or reduce this syndrome.

RESEARCH AREA:	**NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH**
PRINCIPAL INVESTIGATOR:	**Dora Angelaki, Ph.D.**
ORGANIZATION:	**Washington University School of Medicine**
PROJECT TITLE:	**Neural Mechanisms of Vestibular Adaptation**
FUNDING:	**$182,702 (FY 1999); $219,951 (FY 2000)**

PROJECT EXECUTIVE SUMMARY

Changes in vestibular function through disease, trauma and aging occur frequently and are particularly pronounced with exposure to unusual motion or gravitational environments. Throughout the history of the manned space flight program, the introduction of the body into microgravity has produced vestibular-related disturbances that result in personal discomfort and a loss in crew performance. Since the symptoms subside within several days of microgravity exposure, it suggests that the vestibular system responses can adaptively change to altered sensory conditions. These changes may be similar to the process of vestibular compensation, which is observed following unilateral labyrinthine loss or alterations in visual-vestibular interactions.

In order to better understand the nature of vestibular adaptation and its effects upon motor function, the processes underlying neural plasticity and adaptation to altered vestibular signals must be established. The proposed project will utilize systems and electrophysiological approaches to relate stimulus parameters to vestibular adaptation through quantification of the adaptive properties of the vestibulo-ocular reflex and, in particular, the otolith-ocular system in response to changes in the gravitoinertial accelerations (i.e., hypergravity) brought about through centrifugation. Three-dimensional eye movements will be recorded and quantified before, as well as after short-term centrifugal motion. In addition, the underlying neural mechanisms that are responsible for the adaptive behavior will be determined by quantifying the gravity-sensitive properties of behaviorally and electrophysiologically identified vestibular nuclei neurons before and after centrifugation. The adaptive changes in otolith-ocular responses and the associated neural elements needed to be understood in order to provide a functional framework regarding adaptive changes in otolith function not only in microgravity but also in vestibular compensation.

RESEARCH AREA:	NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH
PRINCIPAL INVESTIGATOR:	W. Michael King, Ph.D.
ORGANIZATION:	University of Mississippi Medical Center
PROJECT TITLE:	Signal Processing and Adaptation in Central Otolith Pathways
FUNDING:	$245,487 (FY 1999); $210,022 (FY 2000)

PROJECT EXECUTIVE SUMMARY

The vestibular system detects angular and linear accelerations of the head in space. This information is processed in central vestibular pathways so that it can be used for a variety of important behaviors: regulation of autonomic responses to body position; regulation of postural reflexes and balance; generation of compensatory eye movements; and perceptual awareness of self-motion and orientation. Vestibular dysfunction leads to significant clinical disturbances that can be incapacitating or life threatening. Signal processing underlying angular motion detection by the semicircular canals is well understood. Less, however, is known regarding signal processing of linear motion information by the otoliths, and the effects of dysfunction within this system. Gravity is a linear accelaration, so the otoliths play a crucial role in determining spatial orientation with respect to the earth and in anti-gravity postural reflexes. Dysfunction can cause severe disturbances of balance, autonomic symptoms, and, in the case of pilots or astronauts, life threatening disorientation and space sickness. The fundamental goal of this project is to apply linear systems techniques to the analysis of electrophysiological data. Single unit recordings will be obtained from vestibular neurons in alert, behaving non-human primates during controlled linear motion. The data will be analyzed to learn how central vestibular neurons process afferent signals to extract information about head position and linear translation. This information is essential for understanding the physiological basis for dysfunction of this system. A second goal is to study the interaction of visual inputs with linear motion signals. Sensory conflicts between angular motion and visual inputs have been successfully studied as models of adaptive plasticity, and a similar approach will be employed to study adaptive mechanisms involving linear motion and visual conflicts. This information will be essential to develop treatment strategies for vestibular dysfunction, preventative strategies for at risk subjects such as pilots or astronauts, and to target pharmacological agents to repair or reduce the symptoms of dysfunction.

RESEARCH AREA:	**NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH**
PRINCIPAL INVESTIGATOR:	**Jennifer Raymond, Ph.D.**
ORGANIZATION:	**Stanford University**
PROJECT TITLE:	**Vestibular and Visual Control of Eye Movement**
FUNDING:	**$284,696 (FY 1999); $294,486 (FY 2000)**

PROJECT EXECUTIVE SUMMARY

The vestibulo-ocular reflex (VOR) reduces motion of visual images on the retina by evoking eye movements in the opposite direction to head movements. A form of motor learning, known as VOR adaptation, calibrates the VOR by gradually correcting the reflex when image motion is persistently associated with head turns. VOR adaptation is essential for ensuring adequate visual acuity during head turns and for restoring proper motor and perceptual orientation in space in response to changes in the organism or its environment, such as occur with growth and development, aging, injury to the peripheral or central nervous system, the donning of a new pair of spectacles, or travel in space.

The proposed experiments examine the neural mechanisms of VOR adaptation by asking the following questions: How do the parameters of visual-vestibular stimulation (image motion during head turns) affect VOR adaptation? (Aim 1) What patterns of neural signals are consistently present during stimuli that induce VOR adaptation, and might therefore serve as the neural trigger for VOR adaptation? (Aim 2) How might plasticity in neural pathways through the cerebellum contribute to VOR adaptation? (Aim 3)

The VOR is one of many motor systems that is thought to rely on cerebellum-dependent learning to maintain normal sensorimotor function and for recovery of function following injury. The anatomy and physiology of the cerebellum is very regular across then extent of this structure, therefore, the principles uncovered in studies of VOR adaptation may be useful for the development of rational therapeutic approaches to many forms of sensorimotor dysfunction.

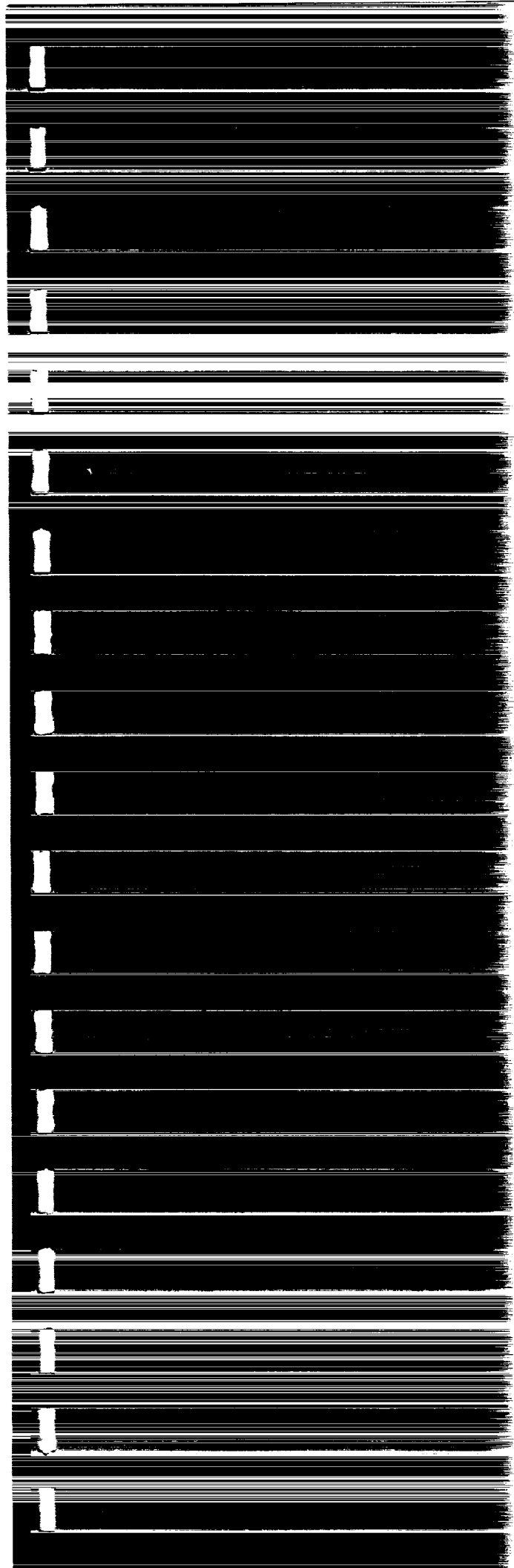
RESEARCH AREA:	**NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH**
PRINCIPAL INVESTIGATOR:	**Helen Cohen, Ed.D.**
ORGANIZATION:	**Baylor College of Medicine**
PROJECT TITLE:	**Management of Adaptation to Altered Sensorimotor States**
FUNDING:	**$107,460 (FY 1999); $117,987 (FY 2000)**

PROJECT EXECUTIVE SUMMARY

Exposure to microgravity causes short- and long-term changes in sensorimotor integration as indicated by astronauts' clinically significant decrements in performance of tasks that require sensorimotor integration shortly after landing. These deficits have potentially serious consequences for crew safety at landing and for crew efficiency in the first few days after landing, either on earth or some other planetary surface. Likewise, patients with vestibularly-related balance impairments have functional limitations in tasks requiring good dynamic balance and visual acuity, with significant implications for their safety and their quality of life. Thus countermeasures, i.e., rehabilitative treatments, are needed for both populations, to facilitate acquisition or reacquisition of adaptive motor strategies in response to sensorimotor challenges. This study will test a rehabilitation approach for maintaining or rapidly reacquiring terrestrial strategies for dynamic balance and sensorimotor integration after exposure to microgravity and for acquiring new movement strategies after loss or impairment of vestibular function. The study will have the following specific aims:

1) Determine the effectiveness of sensorimotor practice variability for transfer of the ability to "learn to learn" adaptive sensorimotor strategies under different conditions of visual/vestibular rearrangement. We will also determine the changes in head-trunk coordination ands lower limb kinematics during gait and determine which kinematic patterns are most compatible with successful motor performance. We hypothesize that subjects trained with variable practice on either visual rearrangement (induced with three sets of experimental lenses) or exercise (with treadmill walking at two speeds) will have significantly better scores than subjects trained in the constant practice groups when post-tested on transfer trials using a fourth set of lenses and a third speed. We further hypothesize that subjects trained with variable practice on both visual rearrangement and exercise will have significantly better scores than subjects in constant practice groups and subjects in groups given variable practice on only one parameter.

2) Determine the effectiveness of sensorimotor practice variability for retention of the ability to "learn to learn" adaptive sensorimotor strategies under different conditions of visual/vestibular rearrangement. We hypothesize that on retention trials given one month after the post-test transfer trials in Specific Aim 1, subjects trained on variable practice on either visual rearrangement or exercise will have significantly better scores than subjects trained in the constant practice groups. Furthermore, subjects trained on variable practice on both parameters will have significantly better scores than subjects in constant practice groups and subjects in groups given variable practice on only one parameter.



NIDCD-NSBRI JOINT PROGRAM FOR THE SUPPORT OF VESTIBULAR RESEARCH

Proposals Received: October 1998
Proposals Selected: September 1999

Year 1 Funding: $1,413 K
(NIH - $1,168 K; NSBRI - $245 K)

Year 2 Funding: $1,342 K
(NIH - $1,132 K; NSBRI - $210 K)

Total Funding: $7,018 K
(NIH - $6,126 K; NSBRI private - $892 K)

1. **Baylor College of Medicine: Helen COHEN, Ed.D.** **Funding:** **Yr. 1: $112 K**
   Yr. 2: $118

MANAGEMENT OF ADAPTATION TO ALTERED SENSORIMOTOR STATES
Co-Is: Jacob Bloomberg, Ph.D. (NASA JSC)

2. **Harvard University: Daniel MERFELD, Ph.D.** **Funding:** **Yr. 1: $299 K**
   Yr. 2: $260 K

DECODING OF GRAVICEPTOR CUES, INCLUDING ADAPTIVE CHANGES
Co-Is: Conrad Wall, Ph.D.
Lionel Zupan, Ph.D.
Robert Peterka, Ph.D. (Oregon Health Sciences U.)
Mark Shelhamer, D.Sc. (Johns Hopkins Univ.)

3. **Stanford University: Jennifer RAYMOND, Ph.D.** **Funding:** **Yr. 1: $287 K**
   Yr. 2: $294 K

VESTIBULAR AND VISUAL CONTROL OF EYE MOVEMENT

4. ***Univ. of Mississippi Medical Ctr: W. Michael KING, Ph.D. Funding:** **Yr. 1: $245 K**
   Yr. 2: $210 K

SIGNAL PROCESSING AND ADAPTATION IN CENTRAL OTOLITH PATHWAYS
Co-Is: Wu Zhou, Ph.D.

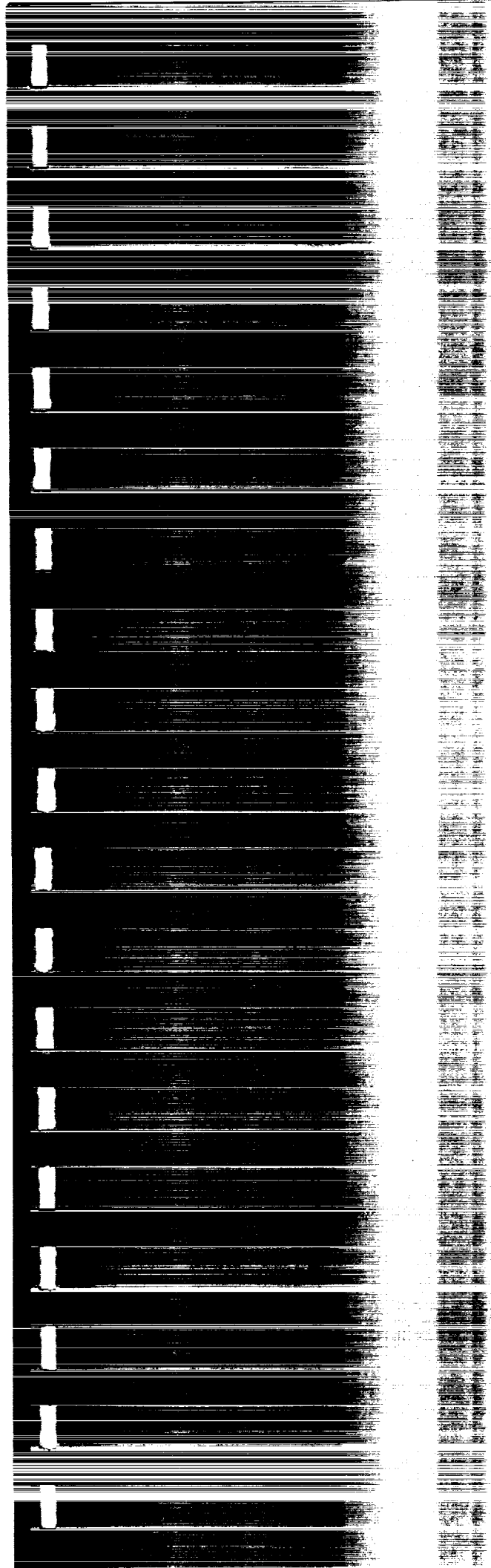
5. **University of Rochester: Scott SEIDMAN, Ph.D.** **Funding:** **Yr. 1: $241 K**
   Yr. 2: $240 K

PLASTICITY IN THE VESTIBULOOCULAR REFLEXES AND PERCEPTION
Co-Is: Gary Paige, M.D., Ph.D.

6. **Wash. Univ. School of Medicine: Dora ANGELAKI, Ph.D. Funding:** **Yr. 1: $229 K**
   Yr. 2: $220 K

NEURAL MECHANISMS OF VESTIBULAR ADAPTATION
Co-Is: J. David Dickman, Ph.D.

***NSBRI supported from private funding sources.**





NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Progress Meeting: Russian Countermeasures Course
Rice University
Fondren Library
Kyle Morrow Room (3rd Floor)
January 17 – 21, 2000

Monday, January 17

8:00 a.m.	Welcome & Introduction Purpose of the Progress Meeting	Drs. Young & Alford
8:30	*Concepts of Biomedical Support During the Piloted Expedition to Mars*	Dr. I. Kozlovskaya (For Dr. A. Grigoriev)
9:30	*The Past, the Present and the Future of the Institute for Biomedical Problems. Prospects for Cooperation.* (by O. Gazenko, A. Grigoriev, and M. Belakovsky)	Dr. M. Belakovsky
10:15	BREAK	
10:30	CARDIOVASCULAR FUNCTION: *Results of Examination of Cardiovascular System of a Man in Long-Term Space Flights*	A. D. Egorov
12:30 p.m.	LUNCH	
1:30	CARDIOVASCULAR FUNCTION: continued	A. D. Egorov
3:30	BREAK	
4:00	CARDIOVASCULAR FUNCTION – DISCUSSION PERIOD	
5:30	ADJOURN	
6:30 – 7:30	RECEPTION – MARRIOTT HOTEL	

Tuesday, January 18

8:00 a.m.	MUSCLES: *Effects of Space Flight Factors On Muscular System*	B. S. Shenkman
10:00	BREAK	
10:15	MUSCLES: (continued)	B. S. Shenkman
12:15 p.m.	LUNCH	
1:15	MUSCLES: DISCUSSION PERIOD	
2:45	ADJOURN	

Wednesday, January 19

8:00	a.m.	BONES: *Results of Studies of Space Flights in Human Skeleton System*	V. S. Oganov
10:00		BREAK	
10:15		BONES: (continued)	V. S. Oganov
12:15	p.m.	LUNCH	
1:15		BONES: DISCUSSION PERIOD	
2:45		BREAK	
3:00		METABOLISM: *The Effects of Space Flights on Metabolism Regulation, Immune and Hematological Systems* (by A. Grigoriev, I. Larina and L. Buravkova)	L. B. Buravkova
6:00		ADJOURN	

Thursday, January 20

8:00	a.m.	METABOLISM: (continued)	L. B. Buravkova
9:00		METABOLISM: DISCUSSION PERIOD	
10:15		BREAK	
10:30		SENSORY-MOTOR FUNCTION: *Results of Studies of Space Flight Effects on Sensory-Motor Functions*	I. B. Kozlovskaya
12:30	p.m.	LUNCH	
1:30		SENSORY-MOTOR FUNCTION: (continued)	I. B. Kozlovskaya
3:30		BREAK	
3:45		SENSORY-MOTOR FUNCTION: DISCUSSION PERIOD	
5:15		ADJOURN	

Friday, January 21

8:00	a.m.	PSYCHOLOGICAL FACTORS: *Results of the Influence of Space Flight Factors on Human Physiological Systems and Psychological Status* (by A. Gerasimovitch, V. Gushin, O. Kozerenko, V. Myasnikov, A. Netchaev, V. Salnitskij, S. Stepanova, E. Shaposhnikov, and O. Shevtchenko. Edited by S. Stepanova)	V. I. Gushin
10:00		BREAK	
10:15		PSYCHOLOGICAL FACTORS: (continued)	V. I. Gushin
12:15	p.m.	LUNCH	
1:15		PSYCHOLOGICAL FACTORS: DISCUSSION PERIOD	
2:45		ADJOURN	

AMENDMENT No. 1

To the Contract Executed on August 9, 1999

between

the National Space Biomedical Research Institute, Houston, Texas, USA

and

the State Research Center of Russian Federation - Institute for Biomedical Problems

This Amendment is to the Contract executed on August 9, 1999 between the National Space Biomedical Research Institute, Houston, Texas, USA (hereinafter referred to as the NSBRI) and the State Research Center of Russian Federation - Institute for Biomedical Problems (hereinafter referred to as the IBMP).

WHEREAS the NSBRI wishes to acquire the scientific report on the effects of space flight factors on human physiological systems and psychological status, and to obtain the IBMP's suggestions of future collaborative activities between NSBRI and IBMP in the sphere of space medicine and biology; and

WHEREAS the IBMP possesses knowledge, experience and documentation in the field of fundamental and applied problems of space biology and medicine, which are based on theoretical and experimental research under ground conditions and aboard spacecraft, as well as practical work related to the medical and biological support of the flight of biological satellites, manned spaceships and stations;

NOW, THEREFORE the NSBRI and the IBMP (hereinafter collectively referred to as the Parties) enter into this Agreement.

ARTICLE 1. SUBJECT OF THE CONTRACT AMENDMENT

The IBMP shall make and submit to the NSBRI a scientific report entitled "Results of Studies of the Effects of Space Flight Factors on Human Physiological Systems and Psychological Status, and Suggestions of Future Collaborative Activities Between the NSBRI and the IBMP, including Joint Works on ISS," hereinafter referred to as the "Report." The Report shall be developed in accordance with the requirements for contents of the report, attached hereto as Appendix 1, and other requirements included in this Agreement.

ARTICLE 2. PREPARATION, REVIEW AND SUBMISSION OF THE REPORT

2.1. All reports, including abstracts, drafts, referances, and figure captions shall be in English.

2.2. The IMBP shall provide abstracts (7-10 pages each, using regular double spaced text including tables and figures) for all six of the issues to be included in the Report and submit these to the NSBRI, to be received by December 23, 1999.

2.3. The IBMP shall prepare complete reports on all six of the issues included in the Report, and present this information at the Progress Meeting described in Article 3.

2.4. The IBMP shall prepare a complete Draft Report, hereinafter referred to as "Draft," in accordance with requirement of Appendix 1, and hand one (1) copy of the Draft over to the NSBRI at the Progress Meeting described in Article 3.

2.5. The NSBRI shall review the Draft provided by the IBMP in accordance with Article 2.3, and provide the IBMP with its comments within four (4) weeks from the date of receipt of the Draft, so that the IBMP can develop the final version of the Report.

2.6. The IBMP shall make necessary changes in the Draft in accordance with the comments of the NSBRI described in Article 2.5, and, within one month from the date of receipt of the comments, will send one (1) copy of the final version of the Report to the NSBRI by express mail.

ARTICLE 3. PROGRESS MEETING

2.1. The Parties shall hold a Progress Meeting in Houston, Texas, USA for five (5) days, from January 17 through January 21, 2000, in order that the NSBRI would have the opportunity of discussing the contents of the Report in detail with the IBMP's experts.

2.2. The NSBRI is responsible for the preparation and operation of the Progress Meeting and has the right to copy and distribute any written materials and recordings of the Meeting.

2.3. The list of the Russian experts who attend the Progress Meeting will be determined by the IBMP and will include eight (8) persons.

ARTICLE 4. FINANCIAL ARRANGEMENTS

4.1. The price for preparation and submission of the Report, except for the travel expenses of the IBMP's experts for the Progress Meeting described in Article 3, is US$100,000 (one hundred thousand US dollars). The price shall be final and definitive except when the Parties agree on a modification of the price reflecting future modifications of the Report described herein.

4.2. The NSBRI shall cover travel expenses (including airfare Moscow-Houston-Moscow, per diem at a rate of US$58 for one person per day, accommodation, and medical insurance) for the eight (8) IBMP experts who attend the Progress Meeting described in Article 3. The details of method of the payment will be otherwise agreed between the NSBRI and the IBMP.

4.3. The payment of the price described in Article 4.1 shall be made as follows :

4.3.1. (1) US$40,000 (forty thousand US dollars) shall be paid within 30 days from the date the conclusion of the Agreement.

(2) US$30,000 (thirty thousand US dollars) shall be paid within 45 days after receipt of the Draft by the NSBRI.
(3) US$30,000 (thirty thousand US dollars) shall be paid within 45 days after receipt of the final version of the Report by the NSBRI.

4.3.2. All payments shall be made by the NSBRI by cable remittance to the IBMP's account No.40502840400000000555 in VNESHTORGBANK, SWIFT VTBRRUMM

or

to the account of the DONAU-BANK AG, Vienna in Credit Lyonnais, New York No. 0100-678000-100 in favour of a/c of VNESHTORGBANK No. 617203-413. Beneficiary: the State Research Center - Institute for Biomedical Problems (76-A, Khoroshevskoye Shosse, 123007, Moscow, Russia), No. 40502840400000000555 in VNESHTORGBANK.

SWIFT DONAU BANK AG, VIENNA: DOBA AT WW
SWIFT Credit Lyonnais, New York: CRLY US-33.

Address of VNESHTORGBANK: 103031
16 Kuznetsky Most,
Moscow
Russia
Phone: +007-095-204-64-40/41/42
Telex: 412362 BFTR RU
Fax: +007-095-956-37-27

4.4. All payments on this Agreement are accepted as net payments in favour of the IBMP without any deductions.

ARTICLE 5: TAXES AND DUTIES

5.1. All duties, taxes and other expenses in connection with this Agreement charged on the territory of the USA, as well as expenses charged in the USA in connection with currency exchange and transfer of all the payments to the IBMP's account above mentioned shall be borne by the NSBRI.

5.2. All duties, taxes and other expenses in connection with this Agreement charged on the territory of Russia, shall be borne by the IBMP.

ARTICLE 6. LANGUAGE

The Report and all related data and documentation to be provided under this Agreement shall be in the English language.

ARTICLE 7. ASSIGNMENT OF CONTACT POINTS

The Parties assign the following representatives as the respective points of contact for the implementation of this Agreement and for receiving all notice and other communications required.

(1) Points of contact for administrative and contractual matters:

The NSBRI: James E. Cooper
National Space Biomedical Research Institute
Phone: 713-798-7412
Facsimile: 713-798-7413

The IBMP: Dr. Mark S. Belakovsky
Head of Department for Dissemination &
Implementation of Achievements in
Space Biology and Medicine
Phone: 007-095-195-15-00,
Facsimile: 007-095-195-22-53

(2) Points of contact for technical and scientific matters

The NSBRI: Ronald J. White, Ph.D.
Associate Director, NSBRI
Phone: 713-798-7412
Facsimile: 713-798-7413

The IBMP: Dr. Inessa B. Kozlovskaya
Head of Department
Phone: 007-095-195-23-75
Facsimile: 007-095-195-22-53

Each Party may change the above individuals by written notice from the signatory of the other Party's signatory.

ARTICLE 8. TREATMENT OF THE REPORT

8.1. The authorship on the Report and all related data and documentation provided by the IBMP to the NSBRI will be reserved by the IBMP experts who are involved in developing the Report.

8.2. The NSBRI will have the rights to copy, distribute, and use the Report and Progress Meeting materials partly or in whole.

8.3. The NSBRI may provide the Report and Progress Meeting materials partly or in whole without any restraint to the researchers, contractors and subcontractors who are engaged in the projects of the NSBRI.

8.4. The NSBRI may use the knowledge acquired from the Report and the Progress Meeting without any restraint and any fee for its work including development of equipment.

8.5. The NSBRI shall make reference to the authorship of the IBMP experts who are involved in the making of the Report when it distributes the Report and Progress Meeting materials, partly or in whole.

8.6. The IBMP and the NSBRI will preserve confidentiality of all written and oral information and data related to this Agreement.

ARTICLE 9: FORCE MAJEURE

9.1. In cases of force majeure circumstances (fire, flood, earthquake, war) which are beyond the Parties control and prevent fulfillment of obligations under this Agreement, timelines for fulfillment of such obligations are appropriately put off in accordance with the duration of force majeure circumstances.

9.2. The Parties will immediately inform each other about the beginning and the end of force majeure circumstances. A certificate given by the Chamber of Commerce of the appropriate country will be proof of the existence of force majeure circumstances. Failure to notify or untimely notification results in the loss of right to refer to any such circumstance as a factor freeing of responsibility for failure to fulfill obligations.

9.3. If force majeure circumstances last longer than three (3) months, any of the parties will be free to cancel this Agreement, notifying the other Party of its intention in writing 30 days before without any material claims to each other.

ARTICLE 10. SETTLEMENT OF DISPUTE

10.1 In case of any dispute and/or differences between the NSBRI and the IBMP resulting from this Agreement or related to its fulfillment, the Parties will take all possible measures to settle them by negotiations.

10.2 If settlement is not reached by them, such dispute shall be finally settled under the Rules of Conciliation and Arbitration of the International Chamber of Commerce by one or more arbitrators appointed in accordance with the said Rules.

ARTICLE 11. OTHER TERMS

11.1. All amendments to this Agreement will be in writing and signed by both Parties.

11.2. This Agreement has been written and signed in two original copies in English. The NSBRI and the IBMP get one copy each.

ARTICLE 12. DURATION

This Amendment will become effective as of the date of the last signature of the Parties and will remain in force through July 31, 2000.

ARTICLE 13. LEGAL ADDRESSES OF THE PARTIES

The NSBRI
The National Space Biomedical
Research Institute
One Baylor Plaza, NA-425
Houston, Texas 77030-3498
Tel: 713-798-7412
Fax: 713-798-7413

The IBMP
The State Research Center of RF
- Institute for Biomedical Problems
Russia
123007, Moscow,
Khoroshevskoye shosse, 76A
Tel.: +007 095195 23 63
Fax: +007 095 195 22 53
Telex: 411048 VUAL SU

IN WITNESS WHEREOF, the Parties have executed this Agreement.

For NSBRI (USA)

Associate Director, NSBRI

Original Signed by 16 December 1999
R. J. White Date

For the IBMP (Russia):

Director

Original Signed by 23 December 1999
A. I. Grigoriev Date

Appendix 1

to Contract Amendment No. 1

Requirements Regarding the Report

"Results of Studies of the Effects of Space Flight Factors on Human Physiological Systems and Psychological Status, and Suggestions of Future Collaborative Activities Between the NSBRI and the IBMP, including Joint Works on ISS"

Contents

1. Sensory-motor functions
2. Cardiovascular system
3. Muscles
4. Bone
5. Metabolism
6. Psychological aspects

Volume

About 400 pages using regular double spaced text, including tables, figures and list of references.

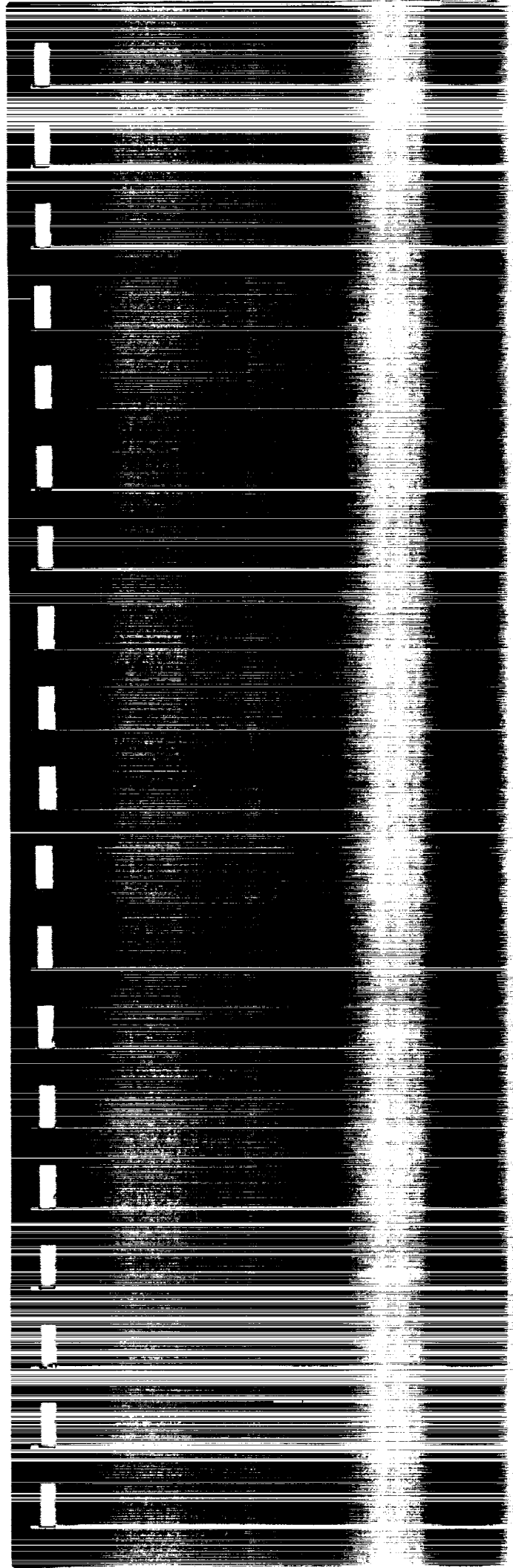
National Space Biomedical Research Institute Publications

Bone Loss Research Team

September 2000

Journal Articles

Allen, M., et al., Ibandronate increases femoral neck bone mineral content, area and ultimate strength in ambulatory hindlimb unloaded rats. *Medicine and Science in Sports and exercise* 32(5 (suppl)): p. S270, 2000.





NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

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September 2000

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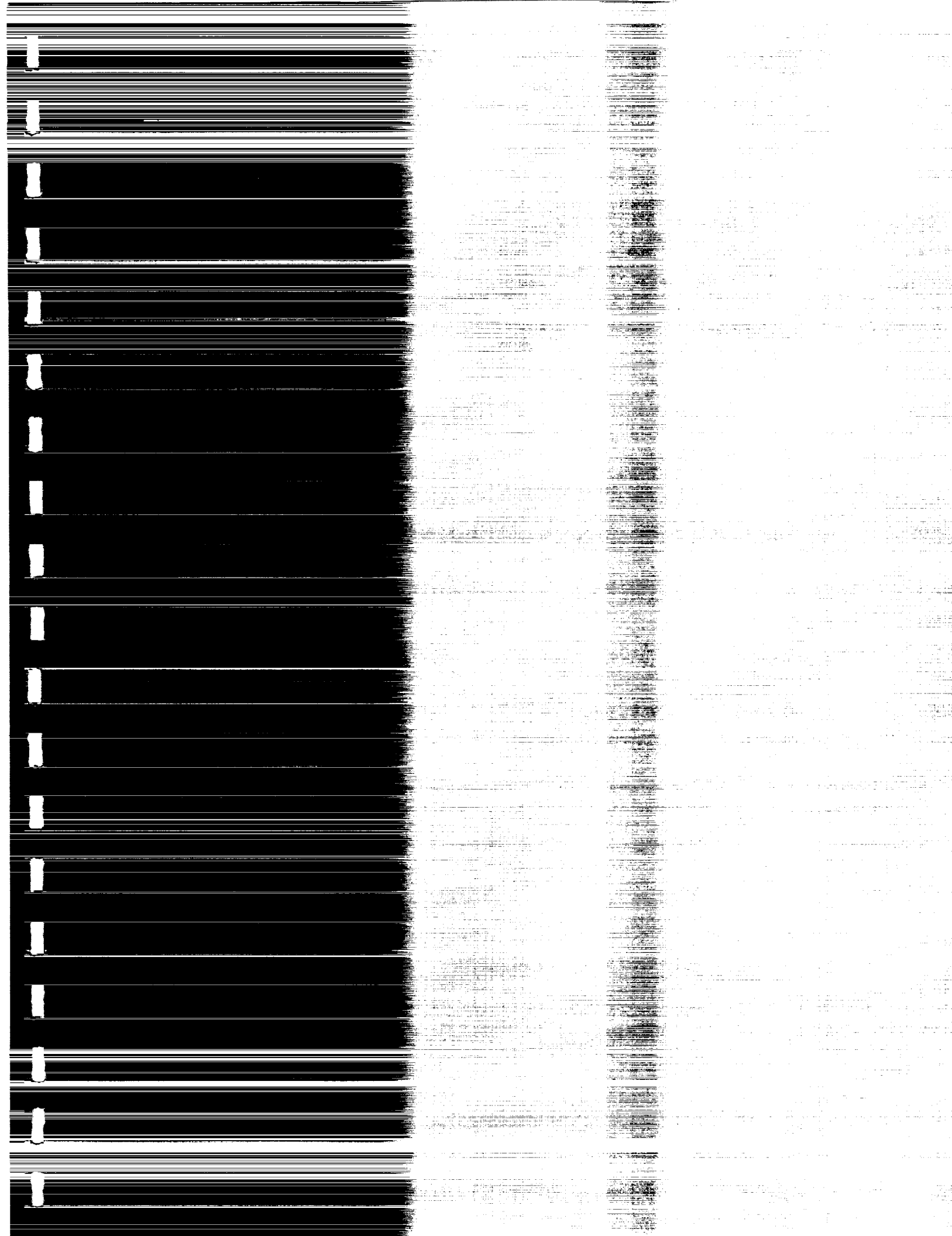
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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Research Announcement

An Opportunity to Participate in the

Core Research Program of the

National Space Biomedical Research Institute

FORMATION OF NEW RESEARCH TEAMS

December 28, 1999
NSBRI 99-02

Letter of Intent Due:	**March 17, 2000**
Proposals Due:	**May 5, 2000**

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Research Announcement

An Opportunity to Participate in the Core Research Program of the National Space Biomedical Research Institute

Formation of New Research Teams

December 28, 1999
NSBRI 99-02

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December 28, 1999
NSBRI 99-02

1.0 OPPORTUNITY

The National Space Biomedical Research Institute (NSBRI), a private, non-profit organization, invites research project applications for the support of ground-based and limited space-flight research in four *new* research areas:

- neurobehavioral and psychosocial factors;
- nutrition, physical fitness and rehabilitation;
- smart medical systems; and
- integrated human function.

The purpose of this announcement is to solicit research proposals from investigators wishing to serve as members of research teams pursuing coordinated programs of activity in each of these four areas. Only investigators interested in becoming members of these four teams should apply to this announcement. Another research announcement recruiting investigators to participate in the eight current NSBRI research areas is expected to be released in February 2000.

Each of the four new teams will consist of a set of individual coordinated and complementary projects focused on a common theme. Team management and coordination will be the responsibility of a program director called a Team Leader. The provisional Team Leaders selected for the organizational phase of the new teams are listed in Section 3 (Table 2).

Applications will be accepted from all categories of organizations, public and private, and for-profit and non-profit, such as universities, colleges, hospitals, laboratories, units of state and local governments, and eligible agencies of the Federal government. The mechanism of support shall be an NSBRI subagreement with funds provided by the National Aeronautics and Space Administration (NASA) through a cooperative agreement (Cooperative Agreement NCC 9-58 with NASA's Lyndon B. Johnson Space Center). Annual renewal awards are subject to an independent, external review. Potential foreign applicants should note that, normally, applications from non-U.S. organizations must be funded by the country of origin, not directly by the NSBRI.

Although space-flight applications may be submitted in response to this announcement, potential applicants should be aware of the limited flight resources available during the time frame of support for flight investigations through this announcement and take those resources into account in preparing their proposal (see Section 4.2).

2.0 BACKGROUND

The NSBRI is responsible for the development of countermeasures against the deleterious effects of long-duration space flight and performs fundamental and applied space biomedical research directed towards this specific goal. Its mission is to lead a world-class, national effort in integrated, critical path space biomedical research that supports NASA's Human Exploration and Development of Space (HEDS) Strategic Plan by focusing on the enabling of long-term human presence in, development of, and exploration of space. This is accomplished by:

- designing, testing and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight;
- defining the molecular, cellular, organ-level, integrated responses and mechanistic relationships that ultimately determine these impediments, where such activity fosters the development of novel countermeasures;
- establishing biomedical support technologies to maximize human performance in space, reduce biomedical hazards to an acceptable level, and deliver quality medical care;
- transferring and disseminating the biomedical advances in knowledge and technology acquired through living and working in space to the general benefit of mankind, including the treatment of patients suffering from gravity- and radiation-related conditions on Earth; and
- ensuring open involvement of the scientific community, industry and the public at large in the Institute's activities and fostering a robust collaboration with NASA, particularly through NASA's Lyndon B. Johnson Space Center.

The NSBRI was established in April 1997 following competitive selection by NASA. Primary support for the NSBRI's activities is furnished by NASA through a cooperative agreement although funds to support Institute activities also come from several sources, including the institutions involved in carrying out the NSBRI's programs. The cooperative agreement award is for a five and one-half year base period, lasting until September 30, 2002, and three five-year optional extensions. Current base funding has been set at approximately $10 million annually. However, NASA has notified the Institute that it would like the NSBRI to expand its activities significantly and will provide additional funds during FY 2000 to develop the infrastructure to support planned program growth beginning in FY 2001. This solicitation is being issued in anticipation of a substantial increase in the NSBRI's core research budget beginning in October 2000, an increase that will require appropriate budgetary authorization and approval by the U.S. Congress. Prospective investigators should be aware that the implementation of the planned augmentation described in this announcement is contingent upon such favorable Congressional action.

2.1 Institute Infrastructure

The NSBRI is governed by a consortium of twelve institutions that includes Baylor College of Medicine, Brookhaven National Laboratory, Harvard Medical School, The Johns Hopkins University School of Medicine and the Applied Physics Laboratory, Massachusetts Institute of Technology, Morehouse School of Medicine, Mount Sinai School of Medicine, Rice University, Texas A&M University, the University of Arkansas for Medical Sciences, the University of Pennsylvania Health System, and the University of Washington. The Institute's headquarters are located in Houston at Baylor College of Medicine.

Because of the nature of the competitive process used by NASA to select the NSBRI, most of the Institute's initial three-year research program is carried out at the consortium institutions. There are, however, no restrictions concerning institutional participation in Institute activity. In fact,

the current program is carried out at more than twenty institutions and government laboratories in addition to the consortium. The management plan for the Institute is based on the model used by the National Institutes of Health. An independent Board of Scientific Counselors is responsible for assuring excellence in the Institute's intramural program through independent external peer review, and an External Advisory Council is responsible for advising Institute management concerning programmatic effectiveness. The NSBRI also has a User Panel of former and current astronauts and flight surgeons responsible for assuring that the research program is focused squarely on astronaut health and safety. An Industry Forum of representatives of space and biomedically-related industries assists the Institute in developing industry participation in NSBRI and in timely technology transfer. In addition to its research program, the NSBRI has developed a vital education and outreach program which takes advantage of the Institute's core research activities.

2.2 Current Research Program

The NSBRI's initial strategic research agenda involves eight teams of scientists focused on:

- *Bone Loss* – Addressing the loss and weakening of bone during space flight with the inherent fracture risks;
- *Cardiovascular Alterations* – Addressing inflight increase of cardiac dysrhythmias and postflight impairment of the cardiovascular response to orthostatic and exercise stress;
- *Human Performance* – Addressing maintenance of high cognitive performance and vigilance despite environmental stress and sleep disturbances;
- *Immunology, Infection and Hematology* – Addressing the potential for immune system impairment and altered susceptibility to infection, increased allergic response, decreased blood volume and postflight anemia;
- *Muscle Alterations and Atrophy* – Addressing the loss of skeletal muscle mass, strength and endurance that accompanies space flight;
- *Neurovestibular Adaptation* – Addressing the problems of space motion sickness and disorientation during flight and the postflight problems of balance and gaze disorders;
- *Radiation Effects* – Addressing the problem of increased cancer risk caused by the natural space radiation environment; and
- *Technology Development* – Developing instrumentation that will enhance the research of the other teams and transferring the technology to industry for the benefit of society.

Each research team consists of investigator groups working on complementary projects focused on a common theme. Team management and coordination is the responsibility of a program director called a Team Leader while overall scientific direction is the responsibility of the Institute Director and Associate Director. The total current intramural research program, including all eight research areas, involves 41 projects, with an average funding per project of approximately $200,000 (Direct + Indirect Costs). Details concerning the current intramural projects and team leaders are provided on the web at: www.nsbri.org/research/newresearch.html.

In addition to this core intramural research program, the NSBRI has developed a joint program with the National Institute on Deafness and Other Communication Disorders (NIDCD) that jointly funds six competitively awarded extramural grants related to the dynamic adaptation of central vestibular function, an area of common interest. Finally, the NSBRI has begun to develop non-U.S. partnerships with the objective of enlarging the core research program by including projects carried out in other countries and supported by those countries. At this time, the Institute has signed an agreement of affiliation with the Institute of Aerospace Medicine of the German Aerospace Center in Cologne (Deutsches Zentrum für Luft- und Raumfahrt e.V., DLR),

an agreement of cooperation with the Institute for Space Physiology and Medicine in Toulouse, France (Institut de Médecine et de Physiologie Spatiales, MEDES), and a framework agreement with the Politecnico di Milano. The NSBRI also has contractual relationships with the Russian Institute for Biomedical Problems in Moscow.

2.3 Planned Augmentation

On the basis of the Institute's initial successes, NASA and the NSBRI developed an augmentation plan that includes an increased number of research areas and intramural teams to allow for more complete coverage of the critical research problems of space biomedical research. It entails increased funding levels for all of the research areas and an augmented extramural grants program (the NSBRI-Federal Cooperative Program) based on the model program developed by the Institute with the NIDCD. In addition, the plan opens the opportunity to participate in the intramural team core research program to any member of the scientific community through the issuance of focused research solicitations. Finally, the augmentation plan will include the development of a small, nationally competitive graduate and postgraduate training program in space-related biomedical research and a significant enhancement to the current education and outreach program of the Institute. To carry out this plan, the Institute has already released an announcement to enlarge the NSBRI consortium (Announcement NSBRI 99-01, May 10, 1999) and has selected five new institutions to add to the consortium, bringing the membership to twelve. This present announcement (Announcement NSBRI 99-02) is to form four new research teams. The third announcement, scheduled for release in February 2000, will allow the eight original research teams to be enlarged.

3.0 SPECIFIC RESEARCH FOCUS

Proposals submitted in response to this announcement MUST address one of the four research areas discussed below. Proposals for research that impacts more than one area should be directed to only one primary research area although a secondary research area may be mentioned for possible further review and consideration. The following subsections are meant to guide the investigator to the key problems and issues that are central to each of these research areas. Innovative approaches to the solution of these problems are encouraged.

3.1 General Information

To carry out the NSBRI's primary mission, that of designing, testing and validating effective countermeasures to address the biological and environmental impediments to human space flight (both within and beyond low-Earth orbit), the NSBRI focuses its research program on the needs of exploration-class space missions. These missions pose the greatest challenge to future space travelers, and meeting their challenge with appropriate countermeasures lies at the core of the NSBRI's responsibility. For planing purposes, a typical Mars-type exploration mission might involve trips of six months to one year each way, with a stay on Mars of one to two years. Effective adaptation, supported by appropriate countermeasures, is critical to a successful mission and to the long-term health maintenance of the astronauts. Potential physiological changes that may occur during prolonged space flight include, among others, significant loss of muscle and bone mass, decreased dietary intake of nutrients, profound metabolic and endocrine alterations, important changes in cardiovascular function and deleterious effects on sensorimotor performance. By addressing long-term missions of this type, increased safety, health and performance will be realized for shorter duration space flights.

Critical Path Roadmap. NASA and the NSBRI have begun jointly to develop a research plan aimed at reducing the biomedical risks of exploration-class missions. Included in this plan is a list of the major human risks involved in such missions and a set of critical research questions associated with those risks. For example, in the radiation research area, the primary risks that have been identified include damage to the central nervous system and carcinoma due to cosmic ray particles and ionizing radiation. A complete list of the exploration-mission risks and the associated critical questions may be found through a web site (http://criticalpath.jsc.nasa.gov/) starting in early 2000. Investigators interested in responding to this announcement should become familiar with these risks and critical questions before preparing their final research plan. In addition, potential applicants should review the 1998 report by the National Research Council's Committee on Space Biology and Medicine entitled *A Strategy for Research in Space Biology and Medicine into the Next Century* (www.nap.edu/catalog/6282.html).

Countermeasure Readiness Levels. Since the NSBRI's primary mission concerns countermeasures, it is important to understand some of the steps involved in effective countermeasure development. These steps are called countermeasure readiness levels and are measured on a scale of 1 to 9, with the higher numbers referring to higher levels of readiness. As Table 1 shows, countermeasure development begins with basic research (levels 1 to 3), moves through countermeasure feasibility and development studies (levels 4 to 6), and ends with countermeasure ground evaluation, flight validation and operational implementation (levels 7 to 9). It is expected that the NSBRI's research program will contain studies ranging from level 2 through level 8, with most tasks ranging from level 3 through level 7.

3.2 Neurobehavioral and Psychosocial Factors

Provisional Team Leader: Nora D. Volkow, M.D.
Brookhaven National Laboratory
(See Table 2)

Astronauts aboard extended-duration missions will endure isolation and confinement in the harsh space environment to a much greater degree than has been experienced previously. Maintaining individual neurobehavioral functioning and group psychosocial effectiveness will be vital to assuring mission success. The Neurobehavioral and Psychosocial Factors Team is focused on research that will ensure that astronaut neurobehavioral health is maintained during prolonged missions, that astronaut performance capability is facilitated by appropriate habitat and human-systems interfaces, and that crew functioning is effectively optimized.

The scope of this research area includes: (1) Identification of the neurobehavioral and psychosocial risks to crew health, safety, well being, performance and productivity during long-duration space missions; (2) Evaluation of the effects of space-related stressors (i.e., habitability constraints, microgravity, radiation, work requirements, sleep deprivation, perceived risks, restricted communication with Earth and boredom) on physiological and psychological functions of individuals and crews; (3) Development of accurate, practical techniques and approaches to monitor behavior and performance capability during missions; (4) Development and validation of countermeasures to manage or mitigate space-related risks to neurobehavioral functions and to enhance health, motivation, safety and performance during such missions; (5) Identification of strategies to maintain motivation and ensure an effective quality of life in space; and (6) Development of procedures to determine optimal leadership style, crew composition, organization and communication with Earth.

Table 1. COUNTERMEASURE READINESS LEVELS

BASIC RESEARCH	1. PHENOMENON OBSERVED AND REPORTED, PROBLEM DEFINED.
	2. HYPOTHESIS FORMED, PRELIMINARY STUDIES TO DEFINE PARAMETERS, DEMONSTRATE FEASIBILITY.
RESEARCH TO PROVE FEASIBILITY	3. VALIDATED HYPOTHESIS, UNDERSTANDING OF SCIENTIFIC PROCESSES UNDERLYING PROBLEM.
	4. FORMULATION OF COUNTERMEASURES CONCEPT, BASED ON UNDERSTANDING OF PHENOMENON.
	5. PROOF OF CONCEPT TESTING AND INITIAL DEMONSTRATION OF FEASIBILITY AND EFFICACY.
COUNTERMEASURE DEVELOPMENT	6. LABORATORY/CLINICAL TESTING OF POTENTIAL COUNTERMEASURE IN HUMAN SUBJECTS TO DEMONSTRATE EFFICACY OF CONCEPT FOR SPECIFIC PROBLEM.
COUNTERMEASURE DEMONSTRATION	7. INTEGRATED EVALUATION WITH HUMAN SUBJECTS IN CONTROLLED LABORATORY CONDITIONS SIMULATING OPERATIONAL SPACE FLIGHT ENVIRONMENT.
	8. VALIDATION WITH HUMAN SUBJECTS IN ACTUAL OPERATIONAL SPACE FLIGHT TO DEMONSTRATE EFFICACY AND OPERATIONAL FEASIBILITY.
COUNTERMEASURE OPERATIONS	9. COUNTERMEASURE FULLY FLIGHT TESTED AND READY FOR OPERATIONAL IMPLEMENTATION.

Within this research area, the following six interrelated themes define the range of factors critical for improving crew health and safety and for optimizing performance capability:

A. Biological mechanisms of neurobehavioral dysfunction. Addresses adverse neurobehavioral events associated with alterations of nervous system function due to prolonged exposure to conditions encountered during long-duration space missions (microgravity or altered gravity, radiation, loss of geophysical cues, isolation, restricted motility, confinement, boredom and stress). The focus is on the nervous system response at the cellular, molecular or organismic level to conditions likely to be encountered during these missions.

B. Motivation, cognition and performance. Concerns the assessment and enhancement of individual information processing, cognitive functioning, motivation and operational performance during space missions.

C. Individual factors. Addresses the individual factors involved in astronaut selection, training and performance, the individual issues related to crew monitoring and psychological support during and rehabilitation following a mission, and the impact of individual factors on strategies to deal with potential psychiatric problems and neurobehavioral dysfunction during a mission.

D. Pharmacology in space. Addresses issues related to the utilization and efficacy of psychoactive and psychotherapeutic agents during space missions, including research into pharmacokinetics and drug bioavailability, changes in the blood-brain barrier, drug interactions, psychological and behavioral side effects of medications and their therapeutic effectiveness.

E. Team and interpersonal optimization. Focuses on issues related to leadership, crew selection and composition for individual space missions, crew functioning during such missions (e.g., training, social interaction, time factors, decision making and error management) and crew-ground interactions. In particular, the study of communication is of particular importance as multinational cooperation assumes a more prominent role in mission design and operation.

F. Organizational, cultural and management factors. Examines the effects of cultures (organizational, professional and national) and management goals, policies and priorities on crew communication, performance, problem solving and, ultimately, health and safety.

Research Questions

The preceding themes are associated with a broad range of research questions that currently exceed NSBRI resources for this area. Therefore, this initial announcement focuses on the following specific research questions related to themes A through D. Many of the research questions related to themes E and F will be covered in future initiatives. For convenience, research questions related to themes A through D have been organized below within four categories: risk assessment, mechanisms and processes, countermeasures, and medical diagnosis and treatment. Many questions cut across the research themes and these categories. Individual project proposals should address the relevant research questions below.

Risk Assessment

- What are the fundamental behavioral and social stressors during long-duration missions that will most likely affect crew performance, both individual and team?
- What model(s) of psychological adaptation in isolated and confined environments will best predict the effect on individual and team performance over the course of the mission?
- What inter-subject and intra-subject factors predict vulnerability to performance failure during prolonged space flight? What factors predict psychological dysfunction for long-term

space missions? How can a measurement system be applied to individuals with different primary languages and cultures?

- What noninvasive measures and techniques can be developed to assess the neurobehavioral, neurobiological, neuroendocrine and/or neuroimmune consequences of stress in individuals? What biological, psychological and social factors can be identified that enable a prediction of individual vulnerability to stress effects?
- What model(s) of behavioral health and task performance best predict and provide guidelines for effective treatment of illness during space flight (e.g., depression, anxiety, trauma and other neuropsychiatric dysfunctions)?
- What are the most common and the most serious neurobehavioral and psychosocial threats (perceived and real) posed to individual astronauts on long missions, and what countermeasures effectively mitigate these threats?
- What techniques and technologies can be developed to objectively evaluate human performance? What are the most effective means to monitor the psychological and neurobehavioral health and well being of astronauts, given that such monitoring should conserve critical resources such as crew time and spacecraft demands (e.g., power usage)? What tools can be developed to monitor astronaut cognitive performance during a mission (i.e., basic information processing such as working memory, focal attention, ability to retrieve information from long-term memory)? What tools can be developed to detect cognitive performance deficits during a mission?
- How can a measurement system provide a means of interpreting the performance information in a manner that indicates the likelihood that an astronaut can successfully perform a specific task? How can a measurement system be used to track and advise regarding the use of countermeasures to enhance performance?

Mechanisms and Processes

- What are the acute and long-term effects of exposure to the space environment on human cognition and performance capabilities, including processes of sensation and perception, mood, learning, vigilance, cognition, problem-solving, decision making and motor skills?
- What are the acute and long-term effects of exposure to the space environment (microgravity, confinement, stress) on the nervous system (at the cellular, molecular or organismic levels) and on related neurobehavioral mechanisms, including neurobiology related to behavior and mood regulation?
- What are the effects of space-related stressors (i.e., radiation, microgravity, work schedules, chronic sleep deprivation, perception of risk, constrained space and motion, continuous noise, boredom/monotony/routine) on central nervous system anatomy, neurochemistry, regulatory behaviors (e.g., appetite, sleep), physiology, neuroendocrinology and neuroimmune interactions, including the effects of simultaneous exposure to multiple stressors as would occur in space?
- What are the effects of space-related stressors on the neurobehavioral functions underlying performance capability, mood and group cohesion?
- What are the acute and long-term effects of exposure to the space environment on human emotion and psychological responses, including emotional reactivity, stress responses, long term modulation of mood and vulnerability to affective disorders?
- What are the impacts of long-duration space flight and microgravity on the pharmacokinetics and pharmacodynamics of drugs intended as countermeasures for the neurobehavioral effects of space flight?
- How do human-machine interactions change over the course of a long-duration mission?
- What are the organizational requirements for support of human performance and the development and maintenance of an optimal behavioral ecosystem in space, including the level of

crew autonomy and the distribution of authority, task scheduling, resource allocation and distribution, and implementation of behavioral countermeasures?
- What are the acute and long-term effects of exposure to the space environment in gene expression in animal models?

Countermeasures
- What countermeasures can be developed to minimize impairment of performance or reduced morale by space-flight-related stressors? What combination of behavioral and physiological countermeasures will optimally mitigate specific performance problems associated with stress during a prolonged mission?
- What pharmacological approaches are effective in improving performance in long-duration space flight? How long do these effects last? What are the secondary effects of countermeasure pharmacology (e.g., hypnotics and anti-nauseants) on performance?
- What countermeasures can be developed to optimize motivation and improve the quality of an astronaut's life during extended space missions?
- What are the best countermeasures for rapidly recognizing and rapidly managing neurobehavioral dysfunction, emotional and stress-related dysfunction, neuropsychiatric dysfunction and social-psychological dysfunction?
- What countermeasures can help crewmembers deal with stressors upon return from space? How can families best re-integrate astronauts after long missions? What are the long-term sequelae of life-changes and other aspects of long-duration space missions?
- What pharmacological approaches can be developed to minimize damage from potential neurotoxic effects of radiation?

Medical Diagnosis and Treatment
- What techniques and methods can be developed for use in flight to effectively monitor and detect neurocognitive difficulties during a prolonged mission?
- What techniques and methods can be developed for use in flight to effectively monitor and detect emotional distress and other forms of neuropsychiatric function during a prolonged mission?
- What training is required by the crew or ground personnel in order to recognize psychological difficulties experienced by crewmembers? What role can telemedicine effectively play in the detection and management of such difficulties?
- What drug delivery systems for neurobehavioral problems are best suited to space missions?

3.3 Nutrition, Physical Fitness and Rehabilitation

Provisional Team Leader: William J. Evans, Ph.D.
University of Arkansas for Medical Sciences
(See Table 2)

This research area focuses on nutrition, physical fitness and rehabilitation, and the interactions among them. Research in this area should utilize an integrative approach to develop broad-based, practical countermeasures appropriate for exploration-class missions. These countermeasures, based primarily on human responses to nutrition and exercise and on sound rehabilitation principles, should focus on removing, or reducing to appropriate levels, deficiencies in human functional ability during space and planetary exploration. Thus, they should prevent the

Table 2. Provisional Team Leaders for the New NSBRI Research Areas

Neurobehavioral and Psychosocial Factors

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progression of functional impairments beyond an acceptable level and enable rehabilitation to a functional status appropriate to the specific challenges of the mission phases as they occur.

For the present announcement, the scope of this research area is limited to proposals that address one or more of the following issues: development of countermeasures with the characteristics discussed above; development of performance-based methods to quantitatively assess countermeasure effectiveness; and evaluation of the effectiveness of existing or new ground-based human models of space flight for simulating integrated, multidisciplinary events.

The following interrelated research topics characterize the scope of this research area:

- **Nutrition.** Addresses: (1) development of mission-phase-dependent nutrient requirements; (2) design of countermeasures to avoid single or multiple, concurrent nutrient deficiencies; (3) nutritional/endocrine interactions and influences on metabolic integrity; and (4) development of approaches to assess nutritional status that relate to functional outcome.
- **Exercise/Physical Fitness.** Addresses: (1) quantification of baseline physiological and physical functions necessary for individuals to perform multiple space operational tasks (e.g., EVA, emergency egress, piloting skills, etc.) involving one or more muscle groups (arms, legs, hands, whole body); (2) quantitative assessment of muscle strength and fatigability of specific muscle groups, maximal oxygen uptake and autonomic vasomotor functions before, during and after space flight; (3) identification of combined dietary and exercise prescriptions to assure physical fitness of individuals for an exploration-class mission; and (4) identification of the physical fitness requirements for operational tasks that may change throughout the duration of the mission, especially following transitions between different gravitational environments.
- **Rehabilitation.** Addresses: (1) identification of the tasks necessary to an exploration-class mission; (2) evaluation of those physical tasks identified for such a mission; (3) study of rehabilitation modalities and interventions necessary to maintain performance in the identified tasks during and following gravitational changes; and (4) determination of how physical modalities (e.g., ultrasound, laser, etc.) used in rehabilitation may be adapted to the space environment for the treatment of injuries.

Research Questions

The following questions are provided to assist the applicant in developing a proposal that is focused on relevant research. They are not complete and project proposals may address other questions fitting within the guidelines above.

- What characterizes the astronaut's physiological adaptations during the different exploration-mission phases, and what are the predictors of metabolic imbalance, musculoskeletal deterioration and sensorimotor dysfunction during such a mission? What is the interdependence of motor performance and exercise capacity on nutritional and hormonal factors?

- What are the most appropriate astronaut studies relevant to this research area that can and should be carried out prior to the introduction of full laboratory capability on the International Space Station?

- What are the interactions among nutrition, combinations of different exercise modes (e.g., resistance and/or aerobic exercise), and the stressful environmental factors (e.g., sleep deprivation, circadian disturbances, intense work schedules) associated with long-term space flight?

- Why does food intake typically decrease during space flight? Research should address the potential impact of appetite control, social/behavioral influences, gastrointestinal function, physical activity and alterations in sleep/circadian cycles.

- What are the multiple systems effects of individual countermeasures that seem to ameliorate some of the adverse physiological effects of space flight? For example, can a resistance exercise training regimen designed to minimize muscle and bone loss during space flight favorably impact cardiovascular mechanisms associated with orthostatic tolerance?

- How much of what kind of periodic exercise pattern, dietary change or rehabilitation strategy is required to maintain and restore normal motor function? Research should take into account the mission-phase changes that occur in the threshold and sensitivity of neural, muscular, cardiovascular and metabolic systems to perturbations that occur during exercise.

- What is the relationship between nutritional requirements and different types of exercises or other countermeasures likely to be used during space flight, given the changes in nutrient intake that may occur during flight? This research may focus on nutritional requirements to maintain nitrogen or calcium balance or to ameliorate losses. For example, research could focus on calcium and protein needs and/or dietary supplements and hormonal interventions. The use of dietary supplements and any drug therapy should also include a strategy to evaluate safety, pharmacokinetics and effectiveness.

- What is the impact of restricted caloric intake on countermeasure effectiveness? (Reduction in caloric intake is routinely reported during space missions and may be secondary to neurovestibular problems and motion sickness.)

- What is the role for nutriceuticals, such as enriched foods and physiologic regulators, in the management of metabolic imbalances?

- How should nutritional and physical status be determined during space flight, including measurement of body mass, determination of surrogate metabolic markers, and development of biosensors and monitoring strategies to enable remote evaluation?

- Do the interactions of muscle-strengthening exercises with other systems, such as cardiovascular and neurovestibular systems, depend more on flight phase or individual adaptability? Can exercises intended to enhance musculoskeletal function also have a beneficial effect on visual-vestibular coordination and on locomotion?

- What are the contributions of reduced gravity, restricted food intake, reduced exercise levels, confinement and isolation on acquiring and maintaining motor skills and performance?

- What is the minimal level or threshold of exercise (intensity, duration and frequency) necessary to maintain or to restore specific functional capability during space flight?

- What countermeasures involving diet, exercise and rehabilitation should be used to maintain health and adequate performance of astronauts during and after an exploration-class mission? (It is likely to be unrealistic to attempt to maintain preflight physical fitness and metabolic function throughout the course of prolonged space flight, and that, in fact, this may be contra-indicated if certain physiological adaptations to space flight are beneficial to the space traveler.)

3.4 Smart Medical Systems

Provisional Team Leader: Jeffrey P. Sutton, M.D., Ph.D.
Harvard Medical School
(See Table 2)

This research area focuses on health maintenance and acute and chronic medical care during exploration-class missions. It includes the development of new and advanced concepts of medical monitoring, diagnostic and therapeutic systems. Such systems could assist in training, decision making and therapeutic intervention. The ultimate goal of this research is to develop a smart, integrated medical system that would assist in the delivery of quality health care on an exploration-class mission. This area includes the following topics:

- New or novel medical and surgical techniques;
- Novel sensor development;
- Advanced drug synthesis and delivery systems;
- Robotic medical assistance systems;
- Automated medical data systems, including reliable patient data acquisition and analysis with minimal crew oversight; and
- Automated decision support, training and diagnostic – therapeutic systems.

The following five interrelated themes characterize the research in this area:

- **Patient monitoring and data management.** Concerns the development of methods and systems for minimally invasive and non-invasive individual patient monitoring and data capture, data communication, display and storage. This activity should be tightly coupled with the development of individualized patient models.
- **Diagnosis.** Addresses the topic of assisted diagnosis and includes the development of methods to provide reliable diagnoses of events during each phase of an exploration-class mission.
- **Treatment.** Focuses on development of novel approaches to treat acute and chronic medical and dental conditions, taking into account the constraints of exploration-class missions.
- **Medical system integration.** Addresses the development of an adaptable, easy to use, unobtrusive, and safe medical and decision support system with intelligent components that will include reliable patient monitoring.
- **Autonomous medical operations.** Concerns the development of: autonomous agents that serve as teacher, diagnostician and guide for medical and surgical therapy; systems and devices appropriate for ongoing medical training and augmentation of both knowledge and skill; and onsite manufacturing ability, allowing medical instruments, pharmaceuticals and biomaterials to be fashioned from primary materials.

Research Topics

The following topics are provided to illustrate the research scope of this area. They are not complete; project proposals may address these and other topics singly or in combination.

Novel sensor systems for monitoring and diagnosis. Sensors for medical use on exploration missions should have low noise/high specificity, low power requirements and minimal use of consumables. They should be modular, evolvable, adaptable, unobtrusive, comfortable, safe and easy to use. Non-invasive or minimally-invasive systems are of special interest, as are sensors that capture data relevant to a broad range of clinical and environmental parameters. Examples may include micro-array sensors for various metabolites, tools for assessing genomic and proteinomic expression, and novel means of tissue monitoring (e.g., optical imaging).

Novel three-dimensional imaging strategies. New devices and approaches are needed to provide improved anatomical and functional definition both in routine monitoring and in acute, critical diagnostic circumstances. Three-dimensional data collected inflight should be readily comparable with ground-based data acquired prior to flight. Research is needed to develop automatic measurement capabilities that routinely pass the data on to physiological modeling and decision support components of the medical care system.

Decision support systems and knowledge bases for diagnosis and treatment. Medical care in a remote, isolated environment will depend on automated and semi-automated processes, alerts and reminders, and methods for aiding human decision makers. This, in turn, requires the ready availability of medical knowledge resources, including textbooks, atlases, guidelines, formularies, diagnostic decision aids and other tools. Methods for organizing and retrieving knowledge relevant to a medical problem, inferring conclusions, developing and customizing diagnostic and treatment plans, and monitoring responses are needed.

Novel therapeutic modalities. Recognizing that facilities and specialty expertise for treating illness and injury will be limited, research is needed to identify alternative approaches that emphasize less invasive therapeutic interventions. Methods and devices that can be used by individuals with limited expertise under adverse space-flight conditions are required. The focus should be on approaches that minimize resource requirements and restore functionality, allowing mission completion.

Remote fabrication and pharmacological production for space. Highly flexible ways to produce biological and non-biological materials, tools and medications will be needed to supply exploration-class missions with additional or unplanned equipment, drugs and prostheses, as they are required.

Intelligent general-purpose reasoner. A valuable computer-based reasoning system (reasoner) would assist in the planning of optimal diagnostic and therapeutic courses of action, predict likely outcomes, and design realistic simulations and training scenarios individualized to a specific person. The reasoner would use comprehensive models to help integrate data, compute diagnostic consequences of the data, and provide real-time inference for emergency interventions and mentoring.

Decision support system for monitoring. Formalized approaches are needed to decide how changes in measured data should be brought to the attention of crew and/or ground support personnel. These approaches require domain-specific models of both short- and long-term

changes in physiological status and status of the crew's environment and life-support systems. Computational techniques are required to direct monitoring systems, allocate monitoring resources across systems, and determine when, to whom and how urgently to report monitoring results. Communication latencies, dropouts and limited bandwidth introduce unavoidable disturbances. System architectures and collaborative approaches therefore need to be developed to support the requirement for shared decision making and data management.

Human-computer interaction techniques. New techniques are required to facilitate monitoring, alerting, mentoring and training. To make the content and procedures of a sophisticated health support system available to crewmembers, the system must be easy to use and employ innovative representations of the data and information. Research is needed to define and refine virtual reality visualization, including innovative user interface technologies for feedback and control.

Intelligent systems for mentoring and training. Supervision of crew-conducted procedures by automated processes can make them safer and more effective. For example, an intelligent mentor system, in the form of personalized avatars for each astronaut, could use personalized models of a patient to guide surgical procedures and prevent errors. Similar capabilities could create personalized training scenarios that would allow a crewmember to practice emergency procedures and plan treatments or surgical procedures. Such a mentor would help prevent errors by providing guidance and feedback from the mentoring system.

3.5 Integrated Human Function

Provisional Team Leaders: James B. Bassingthwaighte, M.D., Ph.D.
and
Martin J. Kushmerick, M.D., Ph.D.
University of Washington
(See Table 2)

This research area seeks to develop a sufficient understanding of human function (from molecules to systems) to enable a reliable evaluation and prediction of an astronaut's safety and functional capacity. The critical path analysis mentioned in Section 3.1 identifies the biomedical risks of long-duration missions, and much of the NSBRI's research effort is aimed at studying the major physiological systems involved in those risks. The Integrated Human Function research area is responsible for supplying the holistic, integrated knowledge necessary for understanding the human as a total organism. This understanding of human function needs to be quantitative and predictive and must be sufficient to enable simulation and planning for adequate and timely responses to challenges *en route* and for successful accomplishment of tasks at destination and return to Earth. It will serve as an integrated repository of knowledge on mechanisms and their coordinated operation in the intact human and as a means for evaluating efficacy of proposed countermeasures.

Realization of this level of understanding will enable medical planning and therapy when needed and analyses of human responses to altered physiology in ground-based research, flight preparations during the mission and rehabilitation on return. Research in this area requires the development of strategies for and implementation of mechanism-based computational models and simulations of human functions. Such software modules should be extensible, reusable, interoperable and retargetable, and they need to be based on cellular, tissue- and organ-level

mechanisms. It should be possible to leverage and exploit existing standards, methods and WEB-supported models and databases such as the High Level Architecture (HLA) for simulations of the Department of Defense, the Common Object Request Broker Architecture (CORBA), the designs incorporated in the Physiome Project and the NLM Visible Human, and the Department of Energy's nascent program on the Virtual Human whose perspective is similar to that of NSBRI but whose targets differ. The goal of this research area is to address the relevant multi-system problems in an integrated manner in space explorations and provide appropriate spin-offs for terrestrial problems. A personalized human model will eventually be developed for use in the Smart Medical Systems described in Section 3.4. Comprehensive individual models of the anatomy, physiology, functional status, medical and environmental history of each astronaut will play a significant role in monitoring, diagnosis, treatment and outcomes prediction, and will allow for training and simulation during long-term missions. This is not an exercise in descriptive mathematics and programming.

Proposals targeted for this research area must demonstrate an integrated computer-based approach to scientific knowledge and information about human function with the underlying cellular, tissue- and organ-level mechanisms. Ultimately this research will produce a "digital human." Such a digital human will be dynamic, quantitative, predictive and integrative. It will be robust in the sense that the generic model will be "tunable" for individual astronauts and useful for analyses of animal experiments. It will synthesize mechanistic information vertically and horizontally, including knowledge from the other NSBRI research teams: digital cells + digital organs + digital systems = digital human. Research may contain hypothesis- or discovery-driven experimental work and computer-based modeling as well as systems for computation and database documentation, but the laboratory elements and computer systems must be fully justified and integrated in the project. Developing a meaningful digital human is clearly a multi-year project, and this research announcement only begins this task. Therefore, applicants might propose an analysis of one or more component cells and organs that are understood well enough to demonstrate clearly the feasibility of the project and outline the path for measuring progress and a plan for extending that analysis into a fully-integrated human system.

Because of the importance of changes to selected systems in the microgravity environment, the focus of proposed projects should be one or more of the following physiological systems: cardiovascular, bone, muscle and sensory-motor systems. One middle-to-long-range goal of this program is the development of a functional model of the human musculoskeletal system. This functional model would be based on an anatomical model such as the Visible Human, with a complete set of bones, soft tissues and muscles defined as to their internal structure, function, regulation and metabolism. This musculoskeletal model will eventually be used to deal with problems related to locomotion, responses to exercise and disuse, metabolism and energetics in the intact human. Other goals of the program are to develop generic models of skeletal and cardiac muscle, eventually suitable for understanding and interpreting data on the short- and long-range effects of exercise, microgravity, prolonged lack of usage, injury, dietary deficiency, etc. These models would be composed of a set of submodels encompassing the features of mitochondrial metabolism, myofilament protein mass and behavior, cellular energetics, substrate usage and regulation, signaling pathways, control of gene expression especially regarding the processes of atrophy and hypertrophy, and endocrine and neural signaling. More near-term metabolic and endocrine models might be focused on the musculoskeletal and cardiovascular systems. An example would be the influences of dietary content (e.g., of fat) on muscle atrophy and hypertrophy, on insulin sensitivity, on the relative usage of carbohydrate, fat and protein metabolism, on the effects of exercise and disuse on insulin-sensitive receptor proteins, growth

hormone, GLUT4, and on the transport capacity of sarcolemma for glucose. In the atrophying heart, there are additional risks of arrhythmia with changes in the ionic, metabolic, pH and calcium balances. Whole-cell and whole-heart modeling are encouraged in the interests of describing and predicting responses to stress, diet, injury and atrophy, both in microgravity and upon reentry into Earth's or Mars' gravitational fields.

The research may be organized to address and integrate certain fundamental physicochemical and biological processes, including:

- Signals and signaling pathways - biochemical, endocrine and electrical;
- Biomechanics and movement;
- Energetics, metabolism and their control mechanisms;
- Mass and energy transport and conservation with fluid, electrolyte and acid-base balances;
- Homeostatic regulation and multilevel control in hierarchical systems; and
- Adaptation and repair mechanisms.

These themes provide a solid basis for horizontal integration across cells, organs and systems. In addition, or alternatively, vertical systems integration (synthesizing molecular, biochemical, cellular and organ subsystems) may be chosen to address how hierarchical organization is achieved mechanistically. Finally, functional organization may be considered, such as:

- Multisystem models;
- Integrated sensorimotor models of performance;
- Tissue repair and long-term function alterations and rehabilitation; and
- Body distribution of exogenous therapeutic agents and endogenous signaling molecules and metabolites.

Research Questions

The first set below consists of generic questions. Following this set, as an example only, are more specific questions focused on the musculoskeletal system. The listed order of the questions does not imply a preconceived level of importance.

General System Questions

- What are the common mechanisms through which altered environments (e.g., microgravity, artificial atmospheres, radiation) change the functioning of multiple cells and organs? What mechanisms are specific, rather than common?

- What are the information and signals passed among cells and systems that result in a systemic response to the environmental alterations in the human response towards maintenance of homeostasis?

- There are many time courses of cellular- and organ-level responses (second to days) to stimuli. What approaches account for short time-constant and long time-constant mechanisms? How would these be integrated to produce oscillatory, stationary and steady-states appropriate for a wide range of physiological times?

- What are the normal physiological operational parameters of the component systems in the organism, the operating points in altered environments, and the magnitude of the functional reserve?

- How does one assess the response of an astronaut to altered environments using models of physiological mechanisms? How can this information be used to assess the efficacy of countermeasures?

- How can the analytical system and modeling approach chosen for your project be translated and transposed to other subsystems and thence to the intact human?

- What criteria and content is needed for a database of the required physiological information? Is it useful to extrapolate from current genomic and proteomic strategies, or are new strategies needed?

Specific Integrated Questions for Muscle and Bone

One way to begin the global task of a whole-body musculoskeletal model of muscles, tendons, ligaments, blood vessels, bones and joints is an analysis of the lower limb, which fatigues easily and atrophies in microgravity.

- What are the forces exerted by muscles on bone in terrestrial activities, and how do these change during activities at near-zero gravity, 0.16 G (Moon) and at 0.37 G (Mars)? How does the energy cost for contractile activity and substrate metabolism change as a function of gravitational load?

- What is the contribution to stored elastic energy in specific muscles and tendons in normal mechanics, and how does this change during adaptation to microgravity? How does one assess changes in the properties of these tissues in musculoskeletal function?

- What changes in gene expression and protein lifetimes govern the loss of mass of bone, heart and skeletal muscle during microgravity? Are these changes with atrophy related to changes in metabolic and energetic fluxes? Do these mechanisms account for the altered performance noted in human bed-rest models and/or in animal hindlimb suspension experiments?

- What are the relationships between force-generating mechanisms in skeletal muscle and changes in the structure and function of ligaments, tendons and bones?

- What is the role that the magnitude and patterns of mechanical stresses play in normal bone and muscle metabolism, on proprioceptive and other sensory signals, and on motor output? How are these effects and mechanisms altered in microgravity?

- Can the proposed analyses and models account mechanistically for the adaptations described from current astronaut flight information or from models of microgravity such as human bed-rest and animal hindlimb suspension?

- Does the analysis and model proposed suggest better or alternative experimental models for human and animal studies than those currently used?

- Can the analysis and model proposed be used to find surrogate measures that correlate with fundamental mechanisms derived from human or animal studies which would be useful for developing and testing countermeasures in human subjects?

4.0 APPLICATION PROCEDURES

4.1 General Instructions

Applications are to be submitted on the grant application form PHS 398 (rev. 4/98). These forms are available electronically from grants.nih.gov/grants/funding/phs398/phs398.html. If you do not have access to the Internet, you may order the forms by calling GRANTSINFO at (301) 435-0714 or sending an e-mail to grantsinfo@nih.gov. Instructions for completing the application are found in the PHS 398 application form.

DO NOT SUBMIT THIS APPLICATION TO THE NIH. INSTEAD, FOLLOW THE SUBMISSION INSTRUCTIONS BELOW. Please direct any questions that you may have concerning this application form to the NSBRI: telephone – 713-798-7412, fax – 713-798-7413.

Submit the signed, original application and twenty-five exact photocopies and twenty-five collated sets of appendix materials, in one package, to:

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
REF: NSBRI 99-02
ONE BAYLOR PLAZA, NA-425
HOUSTON, TX 77030-3498.

Applications must be received before 5:00 p.m. CDT, Friday, May 5, 2000. FAXED proposals are not acceptable, neither are electronic mail (e-mail) responses.

4.2 Special Instructions

Research Area – Each application must address one, and only one, of the four new research areas discussed in Section 3 of this announcement. Applications that impact more than one area should be directed to only one primary research area although a secondary research area may be identified on the application. Submitters are requested to identify the primary and, if appropriate, the secondary, research area in the title blank of Section 2 of the face page of the application form (*Response to Specific Request for Applications or Program Announcement*). The "Yes" and "No" boxes may be left blank.

Potential applicants may contact the provisional Team Leaders identified in Table 2 to assist them in determining which research area is most appropriate to apply to or to discuss the timeliness or relevance of their planned research to the research areas described in this announcement. In addition, ALL countermeasure-related proposals should contain a special statement specifying the countermeasure readiness level of the proposed project (see Section 3.1 and Table 1).

Letter of Intent – To facilitate planning for the review process, investigators are requested to advise the NSBRI of plans to submit a proposal responding to this announcement by sending a non-binding letter of intent to propose by March 17, 2000 to:

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
REF: NSBRI 99-02 – Letter of Intent
ONE BAYLOR PLAZA, NA-425
HOUSTON, TX 77030-3498.

This letter should be limited to two pages or less and should contain the names and institutional addresses of all investigators and co-investigators involved in the project, a descriptive title and the primary research area for which the proposal will be intended.

Duration of Proposed Research – Proposals for ground research may be submitted for a maximum duration of three years funding, with an assumed starting date of October 1, 2000. Space-flight investigations should be proposed for a nominal duration of three years funding, with an assumed start date of April 1, 2001. As stated below, flight investigations will be selected in October 2000 for a brief definition period. Following this definition period, proposals may be declined or selected for funding and assigned to a mission. Although some flight investigations may take longer than three years to complete, investigators are requested to assume their flight studies will be completed by October 2004.

Total Annual Cost – It is expected that the average annual total (direct + indirect) cost of selected proposals will be between $200,000 and $250,000. In general, the annual total cost of a single proposal may not exceed $400,000.

Inclusion of Women and Minorities in Research Involving Human Subjects – The NSBRI has adopted the NIH Policy regarding this matter. Thus, women and members of minority groups and their subpopulations must be included in NSBRI-supported biomedical and behavioral research projects involving human subjects, unless a clear and compelling rationale and justification is provided that inclusion is inappropriate with respect to the health of the subjects or the purpose of the research.

Human Subjects and Vertebrate Animals – For proposals involving human subjects or vertebrate animals, please follow the instructions for grant application form PHS 398 (rev. 4/98). If IRB or IACUC review is pending at the time of submission, follow-up certification of IRB or IACUC approval from an official signing for the applicant organization must be sent to the National Space Biomedical Research Institute at the address listed for proposal submission. The NSBRI will forward this information to the scientific review panel administrator. For a list of information to be included in the follow-up certification, please refer back to the form PHS 398 (rev. 4/98) instruction booklet.

Space Flight Investigations – Proposals for space-flight experiments should be submitted separately from ground-based research proposals and not combined in one package. It should be assumed that flight investigations proposed in response to this announcement will be completed by October 2004, with the space-flight resources available between October 2001 and October 2004. Investigators should note that flight resources on the Space Shuttle for the next few years and during the early phase of the International Space Station are expected to be minimal, and the competition for those resources will be intense. Thus, flight proposals should represent mature studies and be based on compelling evidence from previous flight studies or appropriate ground-based research. Flight experiments normally require limited baseline or control studies on the ground, and these should be included as part of a flight experiment proposal. It should be noted that pre- and postflight studies on crewmembers, even with no inflight data collection or protocol activity, are considered flight experiments and should be proposed as such. Preparatory ground research designed to define a flight experiment should be proposed as a ground-based study.

Investigators interested in proposing flight experiments should refer to the *Space Life Sciences Flight Experiments Information Package, 1999*, issued by the International Space Life Sciences Working Group. This package is available on the World Wide Web at

peer1.idi.usra.edu/peer_review/nra/99_HEDS_03.html.

Section 5.0 of that document concerning international application forms and instructions for proposal preparation should **not** be followed; form PHS 398 should be used instead.

Special Ground Facilities – A variety of special ground research facilities, including centrifuge facilities, bed-rest facilities, etc., are available for use by investigators submitting proposals in response to this announcement. Interested investigators are referred to the *Space Life Sciences Ground Facilities Information Package, 1999*, also issued by the International Space Life Sciences Working Group and available on the World Wide Web at the same site

peer1.idi.usra.edu/peer_review/nra/99_HEDS_03.html.

The NSBRI will negotiate appropriate use of those facilities on behalf of selected investigators, but investigators must include the cost of using these facilities in their proposal.

Special Travel and Reporting Requirements – Principal investigators selected in response to this announcement will be expected to attend two, two-day research team meetings each year at a location to be determined and one annual three- to four-day general investigator workshop/retreat in the Houston, Texas area. Budgets should reflect the costs associated with these meetings and should include a statement indicating that this travel is a special requirement. Selected investigators will become part of the NSBRI's intramural research program and will be expected to provide an annual progress report. Progress is reviewed by the NSBRI's Board of Scientific Counselors. In addition, investigators will be required to provide annual project information for inclusion in NASA's *Life Sciences Program Tasks and Bibliography*.

Data Management Plan – Most data collected through NSBRI support are required to be placed in a central Institute data archive. Investigators should plan for delivering their data to the NSBRI archive and must include the cost of data archiving in their submitted proposal. If selected, a data management plan, including a list of the data products and a schedule for their delivery, must be prepared and submitted to the NSBRI. No additional costs should accompany this plan.

5.0 COMPETITIVE PROCESS

5.1 Review and Selection Process

Applications will be evaluated for scientific and technical merit and for the likelihood that the research proposed will have a significant impact on achieving the goals stated in this announcement. The initial review will be carried out by an appropriate panel of experts convened under the auspices of NSBRI's independent Board of Scientific Counselors. As part of the initial review, all applications will receive a written critique and be discussed by the panel. Only those applications deemed to have high scientific merit will be assigned a numerical score. Applicants will receive a copy of the panel's comments and score as soon as they are available. Those proposals deemed to be in the competitive range for this submission will receive a second-level review by the NSBRI scientific program directors to determine relevancy of the proposed project to the research program in the particular research area under consideration. Applicants should be aware that some meritorious proposals may not be selected for funding. Selection recommendations are prepared by NSBRI management, reviewed by the NSBRI External Advisory Council and approved by the NSBRI Board of Directors. (N.B. The initial review group will also examine the provisions for the protection of human and animal subjects and the safety of the research environment.)

Flight proposals may be selected for a brief definition period during which it will be determined whether or not it is feasible to actually carry out the proposed investigation in space within a reasonable time and what the realistic costs of the proposed study are. Flight proposals may be declined following this definition period.

5.2 Evaluation and Award Criteria

The following criteria will be used in the evaluation:

Significance: Is the proposal responsive to the needs of the NSBRI, as expressed in this announcement? If the aims of the application are achieved, how will scientific knowledge be advanced? What will be the effect of these studies on the concepts or methods that drive this field? What is the likelihood that the proposed research will lead to new countermeasures or tests of the utility of countermeasures?

Approach: Are the conceptual framework, design, methods and analyses adequately developed, well-integrated and appropriate to the aims of the project? Does the applicant acknowledge potential problem areas and consider alternative tactics? Are there strong interdisciplinary components?

Innovation: Does the project employ novel concepts, approaches or methods? Are the aims original and innovative? Does the project challenge existing paradigms or develop new methodologies or technologies? Are novel experimental approaches considered? Do preliminary results support the new approaches?

Investigator: Are the scientists in the project, including collaborators, suitably trained for the proposed work? Is the work proposed appropriate to the experience level of the principal investigator and other researchers (if any)?

Environment: Does the scientific environment in which the work will be done contribute to the probability of success? Do the proposed experiments take advantage of available unique features or facilities or employ useful collaborative arrangements? Is there evidence of appropriate institutional support?

Selection will be based on the merit score awarded by the peer review panel, on the programmatic relevance as determined by NSBRI management, on cost, and, in the case of flight proposals, on the feasibility of actual implementation. For studies involving human subjects, the adequacy of plans to include both genders and minorities and their subgroups as appropriate for the research goals and the plans for subject recruitment and retention will be taken into account.

6.0 SCHEDULE

The following schedule is planned for the formation of new research teams by the National Space Biomedical Research Institute:

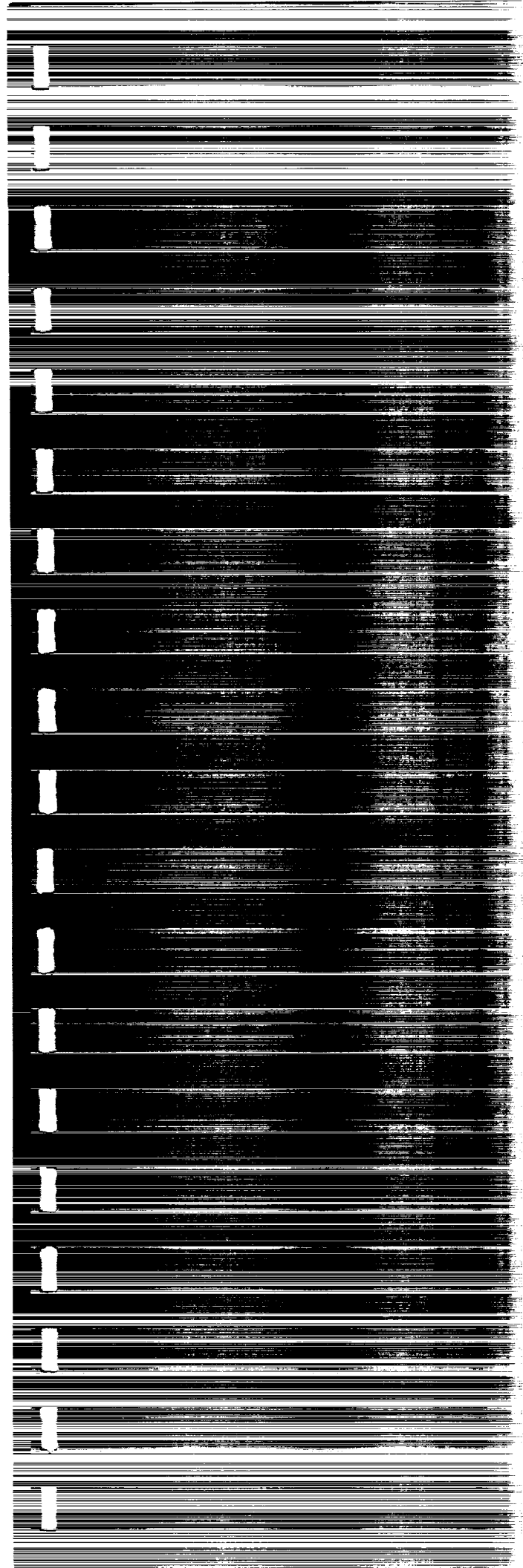
Letter of Intent Due:	March 17, 2000
Proposal Due:	May 5, 2000
Selection Announcement:	August 2000
Funding Initiation:	October 2000

Original signed by
Laurence R. Young, Sc.D.
Director
NSBRI

Original signed by
Ronald J. White, Ph.D.
Associate Director
NSBRI

Original signed by
Bobby R. Alford, M.D.
Chairman of the Board and CEO
NSBRI

December 28, 1999
Date





NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Research Announcement

An Opportunity to Participate in the

Core Research Program of the

National Space Biomedical Research Institute

EXPANSION OF CURRENT RESEARCH TEAMS

February 22, 2000
NSBRI 00-01

Letter of Intent Due:	**April 14, 2000**
Proposals Due:	**June 16, 2000**

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Research Announcement

An Opportunity to Participate in the Core Research Program of the National Space Biomedical Research Institute

Expansion of Current Research Teams

February 22, 2000
NSBRI 00-01

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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Research Announcement

An Opportunity to Participate in the Core Research Program of the National Space Biomedical Research Institute

Expansion of Current Research Teams

February 22, 2000
NSBRI 00-01

1.0 OPPORTUNITY

The National Space Biomedical Research Institute (NSBRI), a private, non-profit organization, invites research project applications for the support of ground-based and limited space-flight research in eight *currently active* research areas:

- bone loss;
- cardiovascular alterations;
- human performance factors, sleep and chronobiology;
- immunology, infection and hematology;
- muscle alterations and atrophy;
- neurovestibular adaptation;
- radiation effects; and
- technology development.

The purpose of this announcement is to solicit research proposals from investigators wishing to serve as members of research teams pursuing coordinated programs of activity in each of these eight areas. Only investigators interested in becoming members of these eight teams should apply to this announcement. Another research announcement recruiting investigators to participate in four new NSBRI research areas was released in December 1999 and is available on the NSBRI web site (www.nsbri.org).

Each of the eight research teams will consist of a set of individual coordinated and complementary projects focused on a common theme. Team management and coordination will be the responsibility of a program director called a Team Leader. The current Team Leaders for these eight teams are listed in Section 3 of this announcement.

Applications will be accepted from all categories of organizations, public and private, and for-profit and non-profit, such as universities, colleges, hospitals, laboratories, units of state and local governments, and eligible agencies of the Federal government. The mechanism of support shall be an NSBRI subagreement with funds provided by the National Aeronautics and Space Administration (NASA) through a cooperative agreement (Cooperative Agreement NCC 9-58 with NASA's Lyndon B. Johnson Space Center). Annual renewal awards are subject to an independent, external review. Potential foreign applicants should note that, normally, applications from non-U.S. organizations must be funded by the country of origin, not directly by the NSBRI.

Although space-flight applications may be submitted in response to this announcement, potential applicants should be aware of the limited flight resources available during the time frame of support for flight investigations through this announcement and take those resources into account in preparing their proposal (see Section 4.2).

2.0 BACKGROUND

The NSBRI is responsible for the development of countermeasures against the deleterious effects of long-duration space flight and performs fundamental and applied space biomedical research directed towards this specific goal. Its mission is to lead a world-class, national effort in integrated, critical path space biomedical research that supports NASA's Human Exploration and Development of Space (HEDS) Strategic Plan by focusing on the enabling of long-term human presence in, development of, and exploration of space. This is accomplished by:

- designing, testing and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight;
- defining the molecular, cellular, organ-level, integrated responses and mechanistic relationships that ultimately determine these impediments, where such activity fosters the development of novel countermeasures;
- establishing biomedical support technologies to maximize human performance in space, reduce biomedical hazards to an acceptable level, and deliver quality medical care;
- transferring and disseminating the biomedical advances in knowledge and technology acquired through living and working in space to the general benefit of mankind, including the treatment of patients suffering from gravity- and radiation-related conditions on Earth; and
- ensuring open involvement of the scientific community, industry and the public at large in the Institute's activities and fostering a robust collaboration with NASA, particularly through NASA's Lyndon B. Johnson Space Center.

The NSBRI was established in April 1997 following competitive selection by NASA. Primary support for the NSBRI's activities is furnished by NASA through a cooperative agreement although funds to support Institute activities also come from several sources, including the institutions involved in carrying out the NSBRI's programs. The cooperative agreement award is for a five and one-half year base period, lasting until September 30, 2002, and three five-year optional extensions. Current base funding has been set at approximately $10 million annually. However, NASA has notified the Institute that it would like the NSBRI to expand its activities significantly and will provide additional funds during FY 2000 to develop the infrastructure to support planned program growth beginning in FY 2001. This solicitation is being issued in anticipation of a substantial increase in the NSBRI's core research budget beginning in October 2000, an increase that will require appropriate budgetary authorization and approval by the U.S. Congress. Prospective investigators should be aware that the implementation of the planned augmentation described in this announcement is contingent upon such favorable Congressional action.

2.1 Institute Infrastructure

The NSBRI is governed by a consortium of twelve institutions that includes Baylor College of Medicine, Brookhaven National Laboratory, Harvard Medical School, The Johns Hopkins University School of Medicine and the Applied Physics Laboratory, Massachusetts Institute of Technology, Morehouse School of Medicine, Mount Sinai School of Medicine, Rice University, Texas A&M

University, the University of Arkansas for Medical Sciences, the University of Pennsylvania Health System, and the University of Washington. The Institute's headquarters are located in Houston at Baylor College of Medicine.

Because of the nature of the competitive process used by NASA to select the NSBRI, most of the Institute's initial three-year research program is carried out at the consortium institutions. There are, however, no restrictions concerning institutional participation in Institute activity. In fact, the current program is carried out at more than twenty institutions and government laboratories in addition to the consortium. The management plan for the Institute is based on the model used by the National Institutes of Health. An independent Board of Scientific Counselors is responsible for assuring excellence in the Institute's intramural program through independent external peer review, and an External Advisory Council is responsible for advising Institute management concerning programmatic effectiveness. The NSBRI also has a User Panel of former and current astronauts and flight surgeons responsible for assuring that the research program is focused squarely on astronaut health and safety. An Industry Forum of representatives of space and biomedically-related industries assists the Institute in developing industry participation in NSBRI and in timely technology transfer. In addition to its research program, the NSBRI has developed a vital education and outreach program which takes advantage of the Institute's core research activities.

2.2 Current Research Program

The NSBRI's initial strategic research agenda involves eight teams of scientists focused on:

- *Bone Loss* – Addressing the loss and weakening of bone during space flight with the inherent fracture risks;
- *Cardiovascular Alterations* – Addressing the inflight increase of cardiac dysrhythmias and postflight impairment of the cardiovascular response to orthostatic and exercise stress;
- *Human Performance Factors, Sleep and Chronobiology* – Addressing maintenance of high cognitive performance and vigilance despite environmental stress and sleep disturbances;
- *Immunology, Infection and Hematology* – Addressing the potential for immune system impairment and altered susceptibility to infection, increased allergic response, decreased blood volume and postflight anemia;
- *Muscle Alterations and Atrophy* – Addressing the loss of skeletal muscle mass, strength and endurance that accompanies space flight;
- *Neurovestibular Adaptation* – Addressing the problems of space motion sickness and disorientation during flight and the postflight problems of balance and gaze disorders;
- *Radiation Effects* – Addressing the problem of increased cancer risk caused by the natural space radiation environment; and
- *Technology Development* – Developing instrumentation that will enhance the research of the other teams and transferring the technology to industry for the benefit of society.

Each research team consists of investigator groups working on complementary projects focused on a common theme. Team management and coordination is the responsibility of a program director called a Team Leader while overall scientific direction is the responsibility of the Institute Director and Associate Director. The total current intramural research program, including all eight research areas, involves 41 projects, with an average funding per project of approximately $200,000 (Direct + Indirect Costs). Details concerning the current intramural projects and team leaders are provided on the web at: www.nsbri.org/research/newresearch.html.

In addition to this core intramural research program, the NSBRI has developed a joint program with the National Institute on Deafness and Other Communication Disorders (NIDCD) that jointly funds six competitively-awarded extramural grants related to the dynamic adaptation of central vestibular function, an area of common interest. Finally, the NSBRI has begun to develop non-U.S. partnerships with the objective of enlarging the core research program by including projects carried out in other countries and supported by those countries. At this time, the Institute has signed an agreement of affiliation with the Institute of Aerospace Medicine of the German Aerospace Center in Cologne (Deutsches Zentrum für Luft- und Raumfahrt e.V., DLR), an agreement of cooperation with the Institute for Space Physiology and Medicine in Toulouse, France (Institut de Médecine et de Physiologie Spatiales, MEDES), and a framework agreement with the Politecnico di Milano. The NSBRI also has contractual relationships with the Russian Institute for Biomedical Problems in Moscow.

2.3 Planned Augmentation

On the basis of the Institute's initial successes, NASA and the NSBRI developed an augmentation plan that includes an increased number of research areas and intramural teams to allow for more complete coverage of the critical research problems of space biomedical research. It entails increased funding levels for all of the research areas and an augmented extramural grants program (the NSBRI-Federal Cooperative Program) based on the model program developed by the Institute with the NIDCD. In addition, the plan opens the opportunity to participate in the intramural team core research program to any member of the scientific community through the issuance of focused research solicitations. Finally, the augmentation plan will include the development of a small, nationally competitive graduate and postgraduate training program in space-related biomedical research and a significant enhancement to the current education and outreach program of the Institute. To carry out this plan, the Institute has already released an announcement to enlarge the NSBRI consortium (Announcement NSBRI 99-01, May 10, 1999) and has selected five new institutions to add to the consortium, bringing the membership to twelve. In addition, the Institute released a research announcement (Announcement NSBRI 99-02, December 28, 1999) to broaden the scope of Institute activity by forming four new research teams. The current research announcement (Announcement NSBRI 00-01) is being released to allow the eight original research teams to be enlarged.

3.0 SPECIFIC RESEARCH FOCUS

Proposals submitted in response to this announcement MUST address one of the eight research areas discussed below. Proposals for research that impacts more than one area should be directed to only one primary research area although a secondary research area may be mentioned for possible further review and consideration. The following subsections are meant to guide the investigator to the key problems and issues that are central to each of these research areas. Innovative approaches to the solution of these problems are encouraged.

3.1 General Information

To carry out the NSBRI's primary mission, that of designing, testing and validating effective countermeasures to address the biological and environmental impediments to human space flight (both within and beyond low-Earth orbit), the NSBRI focuses its research program on the needs of exploration-class space missions. These missions pose the greatest challenge to future space travelers, and meeting their challenge with appropriate countermeasures lies at the core of the

NSBRI's responsibility. For planing purposes, a typical Mars-type exploration mission might involve trips of six months to one year each way, with a stay on Mars of one to two years. Effective adaptation, supported by appropriate countermeasures, is critical to a successful mission and to the long-term health maintenance of the astronauts. Potential physiological changes that may occur during prolonged space flight include, among others, significant loss of muscle and bone mass, decreased dietary intake of nutrients, profound metabolic and endocrine alterations, important changes in cardiovascular function and deleterious effects on sensorimotor performance. By addressing long-term missions of this type, increased safety, health and performance will be realized for shorter duration space flights.

Critical Path Roadmap. NASA and the NSBRI have begun jointly to develop a research plan aimed at reducing the biomedical risks of exploration-class missions. Included in this plan is a list of the major human risks involved in such missions and a set of critical research questions associated with those risks. For example, in the radiation research area, the primary risks that have been identified include damage to the central nervous system and carcinoma due to cosmic ray particles and ionizing radiation. A complete list of the exploration-mission risks and the associated critical questions may be found through a NASA web site (criticalpath.jsc.nasa.gov/). Investigators interested in responding to this announcement should become familiar with these risks and critical questions before preparing their final research plan. In addition, potential applicants should review the 1998 report by the National Research Council's Committee on Space Biology and Medicine entitled *A Strategy for Research in Space Biology and Medicine into the Next Century* (www.nap.edu/catalog/6282.html).

Countermeasure Readiness Levels. Since the NSBRI's primary mission concerns countermeasures, it is important to understand some of the steps involved in effective countermeasure development. These steps are called countermeasure readiness levels and are measured on a scale of 1 to 9, with the higher numbers referring to higher levels of readiness. As Table 1 shows, countermeasure development begins with basic research (levels 1 to 3), moves through countermeasure feasibility and development studies (levels 4 to 6), and ends with countermeasure ground evaluation, flight validation and operational implementation (levels 7 to 9). It is expected that the NSBRI's research program will contain studies ranging from level 2 through level 8, with most tasks ranging from level 3 through level 7.

3.2 Bone Loss

Team Leader: Jay R. Shapiro, M.D.
Uniformed Services University of the Health Sciences
(See Table 2)

Although bone loss has been an important problem since the Skylab flights of the early 1970's, it is only with the advent of extended-duration flights in the Russian Mir program and the availability of increasingly accurate measurements of bone mass in the spine and hip, that the true extent of skeletal loss following prolonged microgravity exposure has been appreciated. Data from Russian and U.S. studies indicate that bone loss proceeds at an average rate of 1-2% per month with a wide range of maximal regional changes, ranging from no loss to as much as 20% loss at specific sites. Although the pattern and extent of bone loss during a prolonged exploration flight can only be estimated, the risks to a flight crew from fracture, soft tissue injury and renal calculus formation constitute real hazards that must be minimized. In addition, another important crew health issue involves the rate of return to baseline bone mass with intact soft tissue integrity

Table 1. COUNTERMEASURE READINESS LEVELS

BASIC RESEARCH	1. PHENOMENON OBSERVED AND REPORTED, PROBLEM DEFINED.
	2. HYPOTHESIS FORMED, PRELIMINARY STUDIES TO DEFINE PARAMETERS, DEMONSTRATE FEASIBILITY.
RESEARCH TO PROVE FEASIBILITY	3. VALIDATED HYPOTHESIS, UNDERSTANDING OF SCIENTIFIC PROCESSES UNDERLYING PROBLEM.
	4. FORMULATION OF COUNTERMEASURES CONCEPT, BASED ON UNDERSTANDING OF PHENOMENON.
	5. PROOF OF CONCEPT TESTING AND INITIAL DEMONSTRATION OF FEASIBILITY AND EFFICACY.
COUNTERMEASURE DEVELOPMENT	6. LABORATORY/CLINICAL TESTING OF POTENTIAL COUNTERMEASURE IN HUMAN SUBJECTS TO DEMONSTRATE EFFICACY OF CONCEPT FOR SPECIFIC PROBLEM.
COUNTERMEASURE DEMONSTRATION	7. INTEGRATED EVALUATION WITH HUMAN SUBJECTS IN CONTROLLED LABORATORY CONDITIONS SIMULATING OPERATIONAL SPACE FLIGHT ENVIRONMENT.
	8. VALIDATION WITH HUMAN SUBJECTS IN ACTUAL OPERATIONAL SPACE FLIGHT TO DEMONSTRATE EFFICACY AND OPERATIONAL FEASIBILITY.
COUNTERMEASURE OPERATIONS	9. COUNTERMEASURE FULLY FLIGHT TESTED AND READY FOR OPERATIONAL IMPLEMENTATION.

Table 2. Team Leaders for the Current NSBRI Research Areas

Bone Loss Jay R. Shapiro, M.D. Walter Reed Army Medical Center Building 2 Ward 64, Rm. 6433 Washington, DC 20307-5001 202-782-8933 202-782-3539 FAX Jay.Shapiro@NA.AMEDD.ARMY.MIL	**Cardiovascular Alterations** Richard J. Cohen, M.D., Ph.D. Harvard-MIT Division of Health Sciences and Technology Massachusetts Institute of Technology 77 Massachusetts Avenue Room E25-335a Cambridge, MA 02139 617-253-7430 617-253-3019 FAX rjcohen@mit.edu
Human Performance Factors ... Charles A. Czeisler, M.D., Ph.D. Brigham and Women's Hospital Harvard Medical School 221 Longwood Avenue, Suite 438A Boston, MA 02115 617-732-4011, ext. 4013 617-732-4015 FAX caczeisler@gcrc.bwh.harvard.edu	**Immunology, Infection and Hematology** William T. Shearer, M.D., Ph.D. Baylor College of Medicine 6621 Fannin, MC 1-3291 Houston, TX 77030 713-770-1274 713-770-7131 FAX wshearer@bcm.tmc.edu
Muscle Alterations and Atrophy	
Susan Hamilton, Ph.D. Baylor College of Medicine, BCMA 429E Houston, TX 77030 713-798-3894 713-798-5441 FAX susanh@bcm.tmc.edu	Robert J. Schwartz, Ph.D. Baylor College of Medicine One Baylor Plaza, BCMC 145EA Houston, TX 77030 713-798-6649 713-798-7799 FAX schwartz@bcm.tmc.edu
Neurovestibular Adaptation Charles M. Oman, Ph.D. Massachusetts Institute of Technology 77 Massachusetts Avenue Room 37-219 Cambridge, MA 02139 617-253-7508 617-253-0861 FAX cmo@space.mit.edu	**Radiation Effects** John F. Dicello, Ph.D. Johns Hopkins University School of Medicine 600 North Wolfe Street Room B1-170 Baltimore, MD 21287-8922 410-614-4194 410-955-3691 FAX diceljo@jhmi.edu
Technology Development Vincent L. Pisacane, Ph.D. Johns Hopkins University Applied Physics Laboratory 11100 Johns Hopkins Road Laurel, MD 20723-6099 443-778-5100 443-778-5995 FAX vince.pisacane@jhuapl.edu	

following the return to Earth's gravity. The Critical Path Roadmap (see Section 3.1) has identified four major bone/connective tissue complications that derive from extended space flight: (1) the development of osteoporosis, (2) enhanced fracture risk and delayed fracture healing, (3) soft tissue injury, and (4) renal calculus formation.

The Bone Loss Team has as its main objectives the development and testing of *countermeasures* to protect astronauts against the above hazards. In considering the design of research projects, applicants should be aware that opportunities for inflight testing of novel countermeasures in animals or humans during the period of this funding cycle are very limited. Therefore, consideration should be given to projects that are primarily designed for initial testing on Earth. In spite of this, research protocols aimed at the development and testing of *operational* countermeasures are strongly encouraged. Developing a basic scientific understanding of the way that microgravity directly and indirectly effects bone, soft tissues and the kidney is key to effective countermeasure development. Thus, the submission of *mechanistic* studies is also strongly encouraged, with the proviso that these be focused on the later development of operational countermeasures that would eventually be tested during space flight.

Within this research area, the following eight interrelated themes define the scope of activities of interest:

A. **Definition of factors related to the maintaining normal bone mass during extended space flight and extraterrestrial habitation.** An important component of this theme is the preflight assessment of the potential for bone loss (baseline bone mass, bone structure and geometry, bone strength, and the genetic influences on the rate of bone loss). In addition, alterations in the mechanical strain environment and in the hormonal environment influence the maintenance of bone mass by impacting mechanisms affecting bone cell biology during exposure to microgravity.

B. **Development of operational countermeasures to bone loss.** These include biomechanical, pharmacologic, nutritional, environmental or other interventions designed to minimize bone loss and reduce the risk of fracture. This theme includes the development of methods to assess the efficacy of countermeasures on bone mineral density and strength and on the structural quality of bone.

C. **Understanding the mechanisms that retard the return of bone mass to preflight levels and the development of countermeasures or specific rehabilitation strategies to promote regaining preflight bone strength.** The mechanisms that retard the response of bone on re-exposure to Earth's gravity are currently undefined. The development of operational countermeasures to minimize fracture risk after return from extended duration space flight is of high priority.

D. **Assessment of fracture risk and the cellular/molecular mechanisms involved in fracture healing.** This theme requires the development of countermeasures (pharmacologic, biophysical, hormonal or nutritional) to promote normal fracture healing in a hypogravity environment. It also includes how microgravity influences the cellular processes involved in fracture healing, the rate of fracture healing and the quality of bone produced. An important issue is the development of suitable animal models to study fracture healing on Earth and during flight

E. **Assessment of the impact of microgravity on the cellular and molecular biology of skeletal and supporting connective tissues.** This theme addresses microgravity-induced changes that lead to a reduction in the quantity, quality and structural integrity of bone and connective tissues. The effects of alterations in mechanical strain and pharmacologic agents on cell structure and function are important components. This includes the development of high throughput models for testing countermeasures including the use of

cDNA microarrays and differential mRNA displays to study gene expression. These applications may help define new drug targets for countermeasure development.

F. **Understanding muscle/bone interactions.** This theme includes the responses of bone and bone-cell populations to progressive muscle atrophy. The regulatory components transducing the effects of mechanical strain on bone also must be defined.

G. **Effects of microgravity on the integrity of supporting connective tissues.** This theme is concerned with the identification and assessment of the extent of injury to intervertebral discs, articular cartilage, meniscus, ligaments and tendons during and after periods of microgravity. Also of concern is how tissue injury inflight predisposes to postflight complications such as osteoarthrosis and disc degeneration.

H. **Minimizing the risk of renal calculus formation.** The risk of renal calculus formation is largely a consequence of increased bone resorption and decreased renal calcium reabsorption. Other microgravity-induced alterations of the urinary environment may increase the propensity to stone formation. Of interest are hypogravity's effect on renal function, the excretion of urinary macromolecular inhibitors/promoters of crystallization environment and nutritional contributions to altered urinary composition. The design of countermeasures should include methods for decreasing the urinary concentration of stone-forming constituents while improving crystallization inhibitory activity.

Research Questions

The preceding themes are associated with a broad range of research questions. For convenience, these have been organized below within five categories. It is emphasized that these topics are intended to focus research efforts on the development of testable and effective countermeasures to skeletal/connective alterations related to human exposure to microgravity.

Prevention of Microgravity-Induced Osteoporosis

- What are the microgravity-induced cellular and molecular changes that lead to reduction in bone quantity and quality?
- What mechanisms underlie the hormonal changes (e.g., PTH, 25(OH)D, 1,25(OH)D2, etc.) during space flight and chronic bed rest?
- What are the important predictors of fracture risk, including ethnicity, gender, bone mineral density, bone biomarkers and bone structural properties?
- Which Earth-based models simulating microgravity are best suited for evaluating countermeasures to bone loss in humans?
- What parameters of mechanical strain (e.g., frequency, intensity, duration) and/or exercise (e.g., mode) influence bone and connective tissues?
- Which nutritional or pharmacologic interventions, together with other modalities (e.g., exercise), best preserve skeletal integrity during exposure to hypogravity?
- How does space flight compromise the interdependence of the neuromuscular and skeletal systems, and to what degree can this be prevented?
- What instrumentation will best predict sequential changes in bone quality and quantity during space flight?

Fracture Risk and Fracture Healing

- Is fracture healing impaired in microgravity?
- Which animal models are best for observing the processes of fracture healing and callus formation in a simulated microgravity environment or during space flight?
- What are the effects of age, gender and race on fracture healing?
- What are the alterations in gene expression during fracture healing in the microgravity environment?
- What is the impact of environmental factors (e.g., sleep, cabin pressure, atmosphere composition) on fracture healing?
- What are the effects of mechanical influences such as fracture immobilization and electrical stimulation on fracture healing?
- What are the effects of growth factors and cytokines on fracture healing?
- Can methods of dynamic modeling be utilized to estimate the long-range effect of prolonged space flight on fracture risk?
- What countermeasures will best accelerate fracture healing in microgravity?

Microgravity and the Cell Biology of Skeletal and Supporting Connective Tissues

- By which mechanisms do bone and cartilage cells (periosteal cells, osteoblasts, osteocytes, osteoclasts and chondrocytes) sense and respond to alterations in gravity?
- How is cell-to-cell communication influenced by microgravity?
- What is cell life span in microgravity?
- What is the importance of apoptosis in modulating the bone's ability to respond to alterations in gravitational force?
- What are the microgravity effects on the maturation of osteoblast and osteoclast precursor cell populations in bone marrow and peripheral blood?
- How will countermeasures influence the processes of bone matrix synthesis, bone resorption and the maintenance of cartilage integrity under microgravity conditions?

Influence of Space Flight on Soft Tissues (Connective Tissues and Cartilage)

- What changes occur in the biology of soft tissue cells *in vitro* and *in vivo* under conditions that simulate aspects of space flight?
- What alterations occur in the molecular composition, microstructure and mechanical properties of the extracellular matrix during space flight?
- To what extent are cellular and extracellular matrix changes occurring during space flight reversible, or compensated for, by postflight therapies? To what extent can these changes be reduced by preflight treatment?
- To what extent are treatment protocols directed at preserving bone also effective in protecting soft tissues?
- How does space flight affect the response of soft tissues to injury and the activation of reparative processes?
- What developments will provide improved diagnostic markers for cartilage degradation and repair?

Renal Stone Formation

- What are the microgravity-induced alterations in serum components, urine formation and urine composition that increase the stone formation risk?
- Are there alterations in the hypothalamic-pituitary renal regulation of salt mineral and water excretion under microgravity conditions that promote kidney stone formation?
- What are the microgravity-induced alterations in urinary macromolecular inhibitors/promoters of stone formation? What is their contribution to risk for stone formation?
- Can nutritional alterations decrease the stone formation risk during space flight?
- Are there effective pharmacologic countermeasures that reduce stone formation risk?
- Can methods be developed for inflight monitoring of stone formation risk?

3.3 Cardiovascular Alterations

Team Leader: Richard J. Cohen, M.D., Ph.D.
Massachusetts Institute of Technology
(See Table 2)

During space flight, the cardiovascular system undergoes adaptive changes in structure and function in response to microgravity and other flight-related factors. While these adaptations appear to be associated with generally adequate cardiovascular performance during short-duration space flight, they are not appropriate upon reentry into a gravitational environment. The extent of cardiovascular adaptation appears to increase with the duration of space flight; however, the extent and implications of these adaptations for long-duration (months to years) space flight remain largely unknown. Space flight is associated with a movement of fluid from the lower extremity to the thorax and head, a modest decrease in intravascular volume, and a modest decrease in arterial pressure. During space flight, the cardiovascular system is not subjected to the stresses associated with changes in posture in a gravitational field. Other physiologic stressors, in addition to microgravity, such as sleep disruption, confinement and other environmental alterations, may adversely affect cardiovascular structure and function. Long-duration space flight leads to the development of orthostatic intolerance upon reentry into a gravitational field, may cause a reduction in cardiac mass, and might alter susceptibility to heart rhythm disturbances. In addition, long-duration space flight affects cardiovascular response to exercise and may, in principle, lead to the manifestation of previously asymptomatic cardiovascular diseases.

The objectives of the Cardiovascular Alterations Team are to: characterize and quantify the adverse effects of space flight on cardiovascular structure and function; determine the mechanisms of these effects; and develop effective countermeasures to reduce these adverse effects to an acceptable level. Proposed research projects may focus on one or more of these three objectives, but applicants should bear in mind that the ultimate goal of the research program is the development of countermeasures to mitigate the adverse effects of space flight on the cardiovascular system.

Research Questions

The research program of the Cardiovascular Alterations Team is driven by the critical risks identified through the Critical Path Roadmap (see Section 3.1). The cardiovascular risks are summarized below, together with some of the research questions whose resolution may lead to mitigation of those risks. The first three risks listed below are accorded the highest research

priority, and the last two are given a lower priority. Research proposed should be directed toward the goal of mitigating one or more of these cardiovascular risks in the most effective way. A number of cross-cutting issues, methods and approaches which may be relevant to the proposed research studies are listed following the five risks.

Impaired Cardiovascular Response to Orthostatic Stress. Upon reentry into the Earth's gravitational field, astronauts experience orthostatic intolerance that limits their ability to function during reentry and after landing. In many cases, the orthostatic intolerance is sufficiently severe that astronauts cannot stand erect for some time after landing. Upon reentry into a gravitational field, blood pools in the dependent arteries and veins which leads to a reduction in the preload to the heart, resulting in a decrease in stroke volume, cardiac output and arterial blood pressure. Factors that may be involved in the development of orthostatic intolerance include structural and functional adaptations of the heart and blood vessels, alterations in volume control mechanisms, alterations leading to an inadequate or defective neural and hormonal regulatory response, alterations in local vascular reactivity, and mechanisms controlling the regional distribution of blood volumes and flows. Orthostatic intolerance represents a current operational problem that may interfere with the astronaut's ability to egress from the spacecraft under emergency conditions, and is a substantial issue with regard to an astronaut's ability to function following a landing on Mars which has a gravitational field 3/8 of that found on Earth. Currently used countermeasures include oral administration of salt and water prior to reentry and application of anti-gravity suits; these countermeasures are not adequate to prevent orthostatic intolerance following long-duration space flight. Investigations elucidating the mechanisms involved in the development of orthostatic intolerance and determining their relative importance are encouraged. In addition, the study of mechanism-based interventions that may serve as countermeasures are particularly encouraged. Studies involving the investigation of factors that affect individual susceptibility to the development of postflight orthostatic intolerance are needed. Such factors may include age, gender and genotype, as well as occupational, physical training and dietary history.

Cardiac Atrophy and Remodeling. Long-term space flight may lead to a measurable reduction in cardiac mass. It is believed that this loss of cardiac mass is associated with cardiac remodeling. It is not known whether these cardiac alterations are reversible and whether they pose a long-term health risk to astronauts. The extent to which cardiac atrophy and remodeling may affect cardiac performance during long-duration space flight is inadequately understood. Furthermore, the detailed mechanisms involved in these changes remain to be elucidated. Research objectives include: quantification of changes in myocyte number, size and geometry; characterization of changes in myocardial matrix and microvasculature; characterization of alterations in myocyte and organ-level mechanical performance; characterization of changes in cardiac gene programming; study of the reversibility and recovery from these alterations; and identification of stimuli and signals that lead to loss of cardiac mass and remodeling. Identification of countermeasures that may prevent these alterations is particularly encouraged.

Occurrence of Serious Cardiac Dysrhythmias. Relatively little data are available on the association of space flight, and in particular long-duration space flight, with the development of heart rhythm disturbances. Anecdotal reports, including one documented 14-beat run of ventricular tachycardia during a Mir mission, suggest that long-duration space flight might lead to an increased incidence of potentially serious heart rhythm disturbances. However, data are currently inadequate to determine whether space flight predisposes the heart to rhythm disturbances. If space flight does significantly decrease cardiac electrical stability, the effects could be catastrophic, potentially leading to sudden cardiac death. In this area, the overriding research

question is whether space flight does increase susceptibility to cardiac dysrhythmias. If space flight is found to increase the risk of cardiac dysrhythmias, then it will be important to determine the mechanisms by which this occurs in order to develop appropriate countermeasures. Potential mechanisms that might lead to a reduction in the stability of the electrical substrate include electrolyte changes, changes in the neural and hormonal milieu, and alterations of cardiac myocytes, myocyte connectivity and extracellular matrix resulting from space flight. Approaches to ascertaining whether space flight might increase susceptibility to serious heart rhythm disturbances may include determining whether changes in cardiac conduction and repolarization that predispose to sustained rhythm disturbances occur in the context of appropriate space-flight models or during space flight itself.

Manifestation of Previously Asymptomatic Cardiovascular Disease. Long-duration space flight may exacerbate previously undetected cardiovascular disease such as coronary artery disease. This area has been assigned a lower research priority than the preceding ones. However, research is needed to determine what procedures should be applied to screen astronauts for asymptomatic cardiovascular disease prior to long-term missions, such as a mission to Mars.

Impaired Cardiovascular Response to Exercise Stress. Long-term space flight may impair cardiovascular response to exercise. However, inflight exercise programs appear adequate to maintain aerobic exercise capacity. This research area is also assigned a lower research priority than the first three areas. In spite of this, research is needed to determine the type, duration and frequency of exercise necessary to maintain cardiovascular system integrity and, potentially, to prevent cardiac atrophy, and to determine whether thermoregulation is impaired during exercise.

<u>Methods, Approaches and Cross-Cutting Issues</u>

- **Experimental Models.** Proposed experimental studies may include ground-based animal or human studies, and space flight studies. As stated in Section 4.2, opportunities for space-flight studies are limited. Because of this limitation, the great majority of experimental cardiovascular research in this area involves animal or human ground-based models of space flight. One question that arises is the extent to which these ground-based models yield results that correspond to space flight. To help validate these models, data available from space-flight studies should be used to evaluate the degree of correspondence with data from ground-based models.

- **Experimental Approaches.** Studies may appropriately involve investigations ranging from the molecular, genetic and cellular level to the organ system level to the entire organism. Studies may include analysis of cardiovascular structure and function, investigation of cardiovascular regulation by local, neural and hormonal mechanisms, and nutritional investigations.

- **Mathematical and Computer Models.** Space flight causes alterations in multiple interacting physiologic systems. The use of mathematical and computer models to elucidate mechanisms, interpret data, and formulate hypotheses to be tested experimentally is encouraged. Such models may include forward models that simulate integrated physiologic behavior or inverse models used to create an individualized model of physiologic function from data recorded on a single individual. Both long-term and short-term phenomena may be analyzed over a variety of length scales from cell to whole organ system.

- **Conditions of Space Flight.** In addition to microgravity, other conditions of space flight,

including sleep disruption, reduced physical stress, environmental factors and psycho-social stresses, may also adversely affect the cardiovascular system.

- **Non-invasive Physiologic Measurement Techniques.** Non-invasive methods for studying the cardiovascular system are needed to help answer research questions and to serve as a means for monitoring astronaut cardiovascular function. Studies that appropriately utilize new, non-invasive (or minimally invasive) measurement techniques are encouraged.

- **Countermeasure Evaluation.** The development of countermeasures should be based on an understanding of the underlying physiologic mechanisms and might include genetic interventions in animal models as proof of concept. Translational research, based on mechanism-based interventions that may serve as countermeasures, is particularly encouraged. Countermeasures may include pharmacological, nutritional and physical interventions (including artificial gravity) and modifications of behavior, activity and environment.

- **Individual Susceptibility.** Investigation of factors that make an individual more susceptible to the adverse effects of space flight on the cardiovascular system may include age, gender, genotype and dietary, occupational and physical-conditioning history.

- **Cardiovascular Rehabilitation.** The adverse effects of space flight may persist following re-entry into a gravitational field. Identification of appropriate strategies for short-term and long-term cardiovascular rehabilitation is needed.

- **Earth Benefits.** Studies of the adverse effects of space flight on the cardiovascular system may also have important implications for clinical medicine issues on earth. Studies which may lead to such benefits are particularly encouraged.

3.4 Human Performance Factors, Sleep and Chronobiology

Team Leader: Charles A. Czeisler, M.D., Ph.D.
Harvard Medical School
(See Table 2)

The success of human space missions depends on each astronaut remaining alert and vigilant while operating sophisticated equipment and procedures. During long-duration space flight, the space environment affects those physiological systems critically involved in human performance, and it is vital to mission success to understand the biological limits of human performance under the space-flight conditions. This team is focused on these issues and, in particular, is concerned with the following aspects of the space environment: microgravity, altered light-dark cycles, altered or reduced sleep/rest opportunities, high levels of automation, and habitation in a remote, inaccessible location. The primary thrust of this team's research program involves altered circadian organization, sleep disruption and cumulative sleep loss, and the associated neurobehavioral decrements occurring during long-duration space flight.

The goals of the Human Performance Factors, Sleep and Chronobiology Team are to understand the basic mechanisms underlying the deterioration of sleep, circadian organization and human neurobehavioral function during space flight and to develop effective countermeasures based on understood mechanisms to optimize sleep, circadian organization and human neurobehavioral function in long-duration space flight. The overall team strategy is described in detail elsewhere (see the web site www.nsbri.org). It includes the following objectives: to assess and monitor the

effects of long duration space flight on sleep, circadian rhythms and human performance; to investigate the mechanisms underlying sleep loss and circadian dysfunction and associated neurobehavioral performance decrements; to develop and validate predictive models for the effects of the space environment and associated sleep and circadian disruption on neurobehavioral performance; and to develop and validate countermeasures to ameliorate performance impairments.

Within this area of research, the following five interrelated themes define the range of factors critical for optimizing human performance capability and improving crew health and safety:

A. **Effects of long-duration space flight on sleep and/or circadian rhythmicity.** This theme addresses the impact of the conditions of long-duration space flight (microgravity, altered light intensity, loss of geophysical cues, isolation, altered physical activity, etc.) on neurobiologic, endocrinological, and behavioral mechanisms (molecular, cellular and organismic) that control sleep and circadian systems.

B. **Effects of sleep loss and/or circadian dysfunction on physical and neurobehavioral performance.** The focus of this theme is to identify the range of acute and chronic adverse effects that sleep loss, sleep disruption, and/or circadian dysfunction have on critical physiologic and performance parameters during long-duration space flight (e.g., neurophysiologic function, physiological alertness, vigilance, cognitive performance, mood/morale, problem solving and communication).

C. **Monitoring and assessment during space flight.** This theme deals with the development of methods for monitoring the status of sleep, sleep homeostasis and circadian organization, as well as technologies that assess and update the current functional status or performance capability of the individual.

D. **Predictive modeling of performance based upon circadian organization and sleep homeostasis.** This theme is concerned with the development of analytical or phenomenological mathematical models that predict individual human performance capability by involving multiple subsystems (e.g., circadian rhythmicity, sleep homeostasis, work-rest schedules, etc.) as an integrated unit across levels of organization, and by estimating the impact of countermeasure use designed to optimize human physical and/or neurobehavioral performance.

E. **Countermeasures**. The research program of this team will not only define the impact of the space environment on sleep and circadian rhythmicity and the effects of the sleep loss and circadian dysfunction on performance but also will develop methods to counter the adverse physiological and behavioral events. These countermeasures may include behavioral, pharmacological, environmental or other adaptive approaches to maintain function and performance under the adverse conditions of long-duration space flight.

Research Questions

The following questions are provided to assist the applicant in developing a proposal that is focused on relevant research. They are not complete, and project proposals may address other questions fitting within the programmatic interests defined above.

- What are the acute and long-term effects of extended duration space missions on biological rhythmicity and sleep, and what is the relationship of these effects to physical and neurobehavioral performance?
- How does space flight or exposure to chronic sleep restriction and/or circadian disruption affect sleep- and circadian-mediated neuroendocrine and autonomic functions, particu-

larly those relevant to risk mitigation (e.g., growth factors, glucocorticoids, monoamines) during extended-duration missions?

- What is the impact of space-flight conditions (e.g., microgravity, altered light exposure and vestibular input, etc.) on the neurobiologic mechanisms (identified at either the cellular, molecular or organismic levels) of sleep-wake regulation, biological rhythmicity and coupling between these and other regulatory functions?
- How can performance during prolonged space flight be optimized by manipulating the neurobiologic processes underlying sleep and/or circadian rhythmicity?
- How do age and gender alter sleep- and circadian-mediated physiologic responses to, and risk mitigation for, prolonged space flight?
- To what extent can the fatigue observed following space flight be due to sleep loss, circadian misalignment, vestibular changes, performance demands or other factors associated with the transition from microgravity to a gravitational environment?
- Do the changes in sleep and/or circadian rhythmicity (e.g., partial chronic sleep deprivation, misalignment of circadian phase) observed during prolonged space flight lead to increased vulnerability to unanticipated operational demands?
- How do environmental factors, such as noise, temperature and/or the intensity and spectral distribution of light, cause or interact with sleep loss and/or circadian disruption to impair neurobehavioral performance?
- What are the appropriate biological model systems for the development and evaluation of countermeasures for sleep and/or circadian rhythm adjustment to long-duration space flight?
- How do task characteristics; operator environments; human-machine interactions; daily, weekly and long-term work-rest schedules; and recovery sleep/naps alter the effects of sleep loss and/or circadian disruption on human performance?
- What are the best methods, in terms of sensitivity and specificity, for monitoring the ongoing status of sleep, sleep homeostasis, circadian regulation and individual performance capability during extended duration space flight?
- How can methods for monitoring and assessment of performance capability during long-duration space flight be effectively implemented given the constraints of space flight (e.g., lack of privacy, transmission delay between the spacecraft and the ground station, etc.)?
- What mathematical models of sleep homeostasis and circadian regulation can effectively predict performance vulnerabilities in individuals subjected to prolonged space flight?
- How can mathematical models of sleep homeostasis and circadian regulation be used to provide guidelines for development and successful implementation of countermeasure strategies?
- How can mathematical and/or empirical models of sleep homeostasis and circadian regulation be used to design improved work-rest and task schedules?
- What measures of sleep, sleep disorders or circadian function predict individual neurobehavioral performance, adaptation or countermeasure efficacy? How can the identification of these parameters be used to facilitate successful adaptation to space flight?
- What are the effects of space flight on the pharmacokinetics, efficacy, side effects and interactions (drug-drug, drug-sleep, drug-circadian) of therapeutic agents designed to improve sleep, circadian regulation, and performance?
- Which behavioral, physiological, pharmacological and/or environmental countermeasures will help crew members reduce disturbances of circadian rhythmicity; sleep disturbances, or homeostatic sleep drive, thereby reducing the associated performance deficits?
- What technological and procedural advances can minimize the probability of error by astronauts whose abilities may be impaired by fatigue or circadian disruption? How can

advances in computer-aided decision making, on-board training or smart check lists be applied to offset cognitive deficits? How can physical ergonomic arrangements be used to mitigate the effect of fatigue in space operations?

- What are the long-term consequences of the use of countermeasures designed to mitigate performance decrements associated with sleep loss and/or circadian disturbances?
- How do countermeasures intended for other physiologic systems (e.g., exercise, activity schedules) interact with sleep, circadian organization, and waking function in long duration space flight? How might the timing of such countermeasure administration be used to improve sleep, circadian organization or waking performance?

3.5 Immunology, Infection and Hematology

Team Leader: William T. Shearer, M.D., Ph.D.
Baylor College of Medicine
(See Table 2)

It has long been known that space flight affects the body's fluids, including blood, in dramatic ways, and it has been suspected that space flight exerts possible harmful effects upon the body's immune system. As the flight duration increases, the risk that crew members will be exposed to infectious agents from other crew members and the spacecraft environment will also increase. Among the possible effects on the immunohematological system are an increase in the susceptibility to infectious diseases and carcinogenesis (see the Critical Path Roadmap, Section 3.1). The factors of space travel that might predispose humans to a secondary state of immunodeficiency disease include stressors, such as psychological and physical stress, isolation, microbial contamination, malnutrition and space radiation.

The current research program has involved the utilization of several Earth-based models, including the study of subjects exposed to the 9-month isolation and exposure of the Antarctic winter, those who are sleep-deprived for several days, and subjects who are enclosed in a self-containment capsule for 6 months. Also, animal research has focused on the antiorthostatic murine model and on isolated tissue and cellular studies. The specific measurements of human immune function and viral reactivation have included those of plasma and cellular cytokines, specific antibody formation, lymphocyte proliferation to recall antigens, and viral DNA quantitation in blood, oral secretions and urine. Animal studies have consisted of measuring several aspects of inflammation and cellular adherence, viral clearance mechanisms and immune responses. Data from these human and animal studies show that stressors (behavioral, environmental, physical and infectious) can modulate the immune response, reactivate latent viruses and enhance the pathogenesis of primary infections by microbial agents. In addition, the hormonal alterations that occur subsequent to an individual's perception of an environmental situation as a stressor interfere with normal pathophysiologic host defense mechanisms (e.g., acute inflammation).

The goals of this research area are to: determine whether immune function will be altered and infections or malignancies increased in astronauts in long-term space travel; determine whether genetic or functional microbial changes impact on the pathophysiology of infections, and develop countermeasures to alleviate the changes in immune and stem cell function, microbial infections and virus reactivations. Eight interrelated themes have been identified in this research area, including the emerging field of stem cell biology as a possible new model system with which to examine the effects of long-duration space travel:

A. **Effects of long-duration space flight upon specific T-cell and B-cell mediated immune responses.** This theme addresses the possibility that secondary immunodeficiency would develop in astronauts on long voyages due to the side-effects of space travel, such as neuroendocrine axis-mediated stress, microbial contamination, malnutrition and solar radiation. Defects in primary and secondary antibody and T-cell responses may be produced by these factors. All of these side-effects are known to negatively influence specific immunity, but systematic studies of sufficient numbers of study subjects are needed to go beyond the experiences previously recorded as anecdotal evidence.

B. **Alterations of cellular and humoral elements of innate immunity in long-duration space flight: cellular metabolism and adhesion molecule biochemistry.** Among the unknowns of space travel is the behavior of the cellular and humoral components of innate immune responses. The migration of leukocytes, cell surface expression and function of adhesion molecules, secretory and phagocytic pathways of neutrophils and monocytes/ macrophages are all subjects that need investigation. In addition, an important aspect of innate immunity requiring study is the healing phase of the inflammatory process following infection or trauma.

C. **Role of mucosal immunity in long-duration space flight.** The mucosal immune system has not been adequately studied in the context of the possible harmful effects of space travel. Using ground-based models of some effects of space flight, it will be possible to systematically examine the elements of mucosal immunity such as viral and bacterial clearance, antigen capture by phagocytes, and generation of neutralizing antibodies, cytotoxic T-cells and other T-cell effector functions.

D. **Microbial infections in long-duration space flight.** A critical question to be addressed is whether the immunosuppressive effects of space travel will lead to acute microbial infection, chronic viral infection and development of malignancy. Based upon the models of primary immunodeficiency, transplantation and AIDS, it is to be expected that given enough space-related immunosuppression, viruses such as Epstein-Barr virus will be reactivated and may produce untoward consequences.

E. **Effects of the space environment on microorganisms and their host interactions.** These would include the pathogenesis of opportunistic infections by bacteria, fungi and viruses. Little is known about the space environment's effects on microbial processes (e.g., secretion, adherence, virulence) and adaptation which may lead to increased pathogenesis. Even a normal immune system may not be able to cope with these space-adapted microbes.

F. **Development of autoimmune disease in long-duration space travel.** As the consequences of disturbing the normal balance of immune system components may be the development of autoimmune responses and disease, it will be important to check for autoantibody production and immune complexes in humans exposed to space travel. Human and animal ground-based models of space flight may be utilized to evaluate this possibility.

G. **Effects of long-duration space flight upon stem/progenitor cell biology and function.** This theme addresses the concern that long-term space travel will adversely affect stem cell biology and development. Stem cells play a critical role in the lifelong regeneration of many organs, including those of the hematopoietic, gastrointestinal, musculoskeletal and nervous systems. Long-term exposure to radiation, in particular, is a concern since the potential for mutagenesis and consequent failure of development or malignant transformation are real possibilities. In addition, it is also possible that known or unknown alterations of cytokine production, adhesion receptor display and signal transduction pathways induced by radiation, stress, infection and/or nutritional deprivation might adversely affect long-term stem cell functioning. Accordingly, these issues are also

worthy of investigation. Use of in vitro culture systems, animal transplantation models and molecular informatics are among the appropriate tools that may be used to address these critical questions.

H. **Countermeasures.** This theme is concerned with the development of specific countermeasures for the possibilities of altered immune responses, viral reactivation and increased infections by other microbial agents in space flight and their consequences. These countermeasures may include immunotherapeutic, pharmacologic, behavioral, environmental, dietary, physiologic and adaptive approaches to prevent the possible harmful effects of long-term space travel upon human immunity and infection.

Research Questions

The following questions are provided to assist the applicant in developing a proposal that is focused on relevant research. They are not complete, and project proposals may address other questions fitting within the guidelines above.

Do factors associated with space flight (e.g., physical and psychological stress, environment, microgravity, nutritional status, radiation, confinement, sleep deprivation, fluid shifts) affect one or more of the following?

- humoral or cell mediated immune function
- innate immune factors
- mucosal immunity
- tolerance or immune surveillance
- reactivation of latent viruses
- susceptibility to microbial infections
- cancer risk
- allergy and hypersensitivity
- response to bacterial, viral or fungal agents; or to factors released from the agents (e.g., endotoxin)
- stem cell/progenitor cell biology and function
- wound healing
  - Do alterations in these systems (areas) pose significant risks to crew members in a manner that exposes then to unacceptable medical risks?
  - Are there assays that reliably predict changes in these systems, and can these assays be adapted to space travel?

- What specific infectious agents will crew members be exposed to and what are their sources?
  - What diagnostic and environmental monitoring needs to be developed?
  - Do space flight conditions alter growth rates, mutation rates or pathogenicity of microorganisms?
  - Do space flight conditions affect reactivation and shedding of latent and persistent viruses?
  - Are there potential alterations in host – microbe balance (e.g., do microfloral agents change over time during space flight)?
- Do unique environmental factors inside the spacecraft promote the transmission and activity of microbial pathogens or cause increased risk of infection, allergy or hypersensitivity reactions independent of altered immune function?
- Can additional new ground-based models be developed to simulate stressors of space flight conditions?
- Are there countermeasures that would prevent the specific changes associated with space flight (e.g., related to malnutrition, psychological stress, viral reactivation, microbial infections, immune alterations, autoimmunity, wound healing, stem cell function and development)?

3.6 Muscle Alterations and Atrophy

Team Leaders: Susan Hamilton, Ph.D.
and
Robert J. Schwartz, Ph.D.
Baylor College of Medicine
(See Table 2)

Exposure to reduced gravity during space travel profoundly reduces the loads placed on muscle. Astronauts lose muscle mass and strength while in space, and, therefore, their ability to function efficiently upon re-exposure to gravity is reduced. Reloading of muscle can also produce muscle injury. Individuals with weaker muscles are less likely to survive a life-threatening emergency requiring muscle strength. Similar wasting of muscles can arise on Earth with prolonged bed rest, limb immobilization, severe burns, malnutrition, nerve injury, motor neuron diseases, cancer, HIV infection and aging.

The research program of the Muscle Alterations and Atrophy Team is driven by the following critical risks identified through the Critical Path Roadmap (see Section 3.1):

- Loss of skeletal muscle mass, strength and/or endurance;
- Inability to adequately perform tasks due to alterations in motor performance, muscle endurance, and structural and functional properties of the musculoskeletal system;
- Propensity to develop skeletal muscle injury, connective tissue dysfunction and bone fractures due to debilitation or maladaptation of the neuromuscular system; and
- Potential impairment of other organ systems secondary to the changes in skeletal muscle.

The primary goals of the team are to develop countermeasures that reduce the above risks and to optimize diagnostics and therapeutic interventions to ensure crew safety, well-being, and performance in changing environments. Attaining these goals will allow humans to live and work in microgravity for extended duration and will minimize neuromuscular injury associated with readaptation to Earth's gravity. To accomplish these goals, the team will elucidate the basic mechanisms involved in microgravity-induced muscle atrophy and, simultaneously, develop countermeasures to regulate these changes.

The development of the most effective countermeasures requires delineation of the signal transduction pathways altered by removal of gravitational load. Most mechanistic insights about the probable causes of muscle atrophy during space flight will initially be obtained from animal and human ground-based models. Where appropriate and feasible, pre- and postflight, noninvasive methods may be applied to the study of these mechanisms in astronauts. Experiments performed in animals will provide guidelines for design of human experiments and will provide molecular/biochemical markers of muscle atrophy. Ground-based longitudinal experiments on human subjects will use several approaches (e.g., bed rest, immobilization, single limb unloading) to address related mechanistic questions. These human studies should facilitate the design of flight experiments by demonstrating ranges of individual characteristics and sensitivities to adaptive interventions and countermeasures, which will guide refinement of testable hypotheses.

Research Questions

The following interrelated questions are provided to assist the applicant in developing a proposal that is focused on the critical factors within the scope of this research area. As stated earlier, this

program will emphasize both applied and basic mechanistic approaches for the development of effective countermeasures. The questions are neither prioritized nor complete. Project proposals may address other questions fitting within the programmatic interests defined above.

- What signaling pathways are altered by load, and how do these pathways cross talk to direct an integrated response?
- What are the targets of the signaling pathways pertinent to the phenotypic response to altered functional demand?
- How are proteolytic pathways (e.g., calpains, caspases, ubiquitin-proteasome) involved in protein degradation processes altered in unloading-induced atrophy?
- Are pro-apoptotic pathways activated in altered loading conditions?
- How are the proliferative and differentiation processes of satellite cells affected by altered loading?
- What are the molecular mechanisms that regulate muscle fiber phenotype with altered load?
- What is the molecular mechanism by which skeletal muscle fibers adjust their length (sarcomere assembly and disassembly) in response to an altered functional working range of the muscle?
- What changes occur at the level of protein and gene expression in response to altered muscle loading and countermeasures?
- What are the mechanisms that regulate the changes in the structural, biochemical and functional properties of the neuromuscular tissue in response to altered load?
- What changes occur in muscle metabolism, composition and energetics in response to altered muscle loading and countermeasures?
- How do soluble factors, such as cytokines, growth factors and hormones contribute independently or synergistically to the response of muscle to altered loading?
- How do influences extrinsic to skeletal muscle systems (such as radiation, stress, and altered fluid and hemodynamic balance) influence the ability of muscle to function in and recover from altered load?
- How do fluid redistribution and altered circulation accompanying exposure to microgravity affect the metabolism, clearance and activity of muscle anabolic and catabolic agents?
- What are the effects of muscle responses to altered load on other body systems (e.g., endocrine, bone, cardiovascular, neurovestibular)?
- What structural, biochemical and functional changes in sensory or motor neurons result from, or act to modify, muscle responses to altered loading?
- By what mechanism does the physical inactivity of skeletal muscle produce a systemic metabolic dysfunction (e.g., metabolic/insulin resistance syndrome or syndrome X)?
- How are low- and high-intensity work-induced muscle fatigue altered in a real or simulated space-flight environment (that includes unloading, radiation, temperature and other factors)? What are the mechanisms to explain these alterations?
- What are the mechanisms through which muscle injury, repair or regeneration occurs following alterations in loading? How can countermeasures positively influence these processes?
- What are the optimal countermeasures for maintenance of muscle and bone structure and function?
- What interventions can precondition and/or rehabilitate muscle to changes resulting from altered loading?
- How can concepts derived from animal models be confirmed in humans and applied to optimize human performance in space?
- How can ground-based models (e.g., human bed rest; limb unloading in humans and animal models) be used to evaluate and optimize countermeasures?

- Do hormonal, pharmacological, gene therapy and nutritional interventions used as countermeasures increase the efficacy of exercise protocols in maintaining muscle mass and function? To what extent?
- What nutritional interventions are needed to minimize losses in muscle mass, strength and endurance?
- How much individual variability is there in baseline values for any given muscle phenotype and in the response of that phenotype to the proposed countermeasure?
- To optimize selection of persons most resistant to the negative consequences of microgravity on muscle, what variables might be considered to pre-screen potential astronauts for long duration space flight?
- Can new countermeasures be found by developing and applying high throughput screens or other novel approaches that capitalize on advances in understanding the mechanisms underlying unloading atrophy or aberrant muscle repair?

3.7 Neurovestibular Adaptation

Team Leader: Charles M. Oman, Ph.D.
Massachusetts Institute of Technology
(See Table 2)

The neurovestibular problems associated with space flight (e.g., space motion sickness, disorientation, oculomotor deficits, postflight postural instability and gait ataxia) typically arise when astronauts transition from 1-G to 0-G, just when their physical and cognitive performance is critical for mission success and safety. Similar problems are expected on exploration-class missions when astronauts make the transition from 0-G to partial G, or from 0-G to an artificial gravity environment. During the shuttle era, space neurovestibular research focused on understanding the effects of unweighting of the otoliths on the vestibulo-ocular reflex (VOR) and on predicting space sickness susceptibility. NSBRI's current neurovestibular research program is investigating context specific pre-adaptation, preflight visual orientation and 3-D spatial memory training countermeasures and developing ways to improve our assessment of postflight posture, locomotion and gaze control problems. Future program expansion will include several new areas of emphasis, including artificial gravity, postflight neurovestibular rehabilitation, improved anti-motion sickness drugs and other areas defined below.

The neurovestibular adaptation research program is aimed at developing scientifically-based countermeasures against the vestibular problems associated with space flight and addresses five major space neurovestibular risk areas identified through the Critical Path Roadmap (see Section 3.1):

- **Disorientation and reduced performance on cognitive and physical tasks**, including vehicle egress, especially during/after G-level changes (associated with acute-spontaneous and head-movement-contingent vertigo, nystagmus, oscillopsia, saccadic errors, reduced dynamic visual acuity).
- **Impaired neuromuscular coordination and/or strength** (gait ataxia, postural instability).
- **Impaired cognitive and/or physical performance** due to spatial disorientation, motion sickness symptoms or treatments (including short term memory loss, reaction time changes, drowsiness, fatigue, torpor, irritability, ketosis) as a result of changes in g-level, or use of artificial gravity.
- **Autonomic dysfunction** (including cardiovascular, respiratory, gastrointestinal, sleep and mood changes) which may be of vestibular origin.

- **Permanent impairment of orientation or balance function** due to microgravity or radiation (causing chronic imbalance, gait ataxia, vertigo, chronic vestibular insufficiency, poor dynamic visual acuity).

The goals of the program are to develop countermeasures that ultimately will to allow crewmembers to: avoid disorientation, meet the physical requirements of emergencies, treat motion sickness without side effects and safely control vehicles and systems. Nine interrelated countermeasures-oriented themes define the scope of this research area:

- Adaptive generalization and context-specific adaptation;
- Artificial gravity;
- Visual (multisensory) orientation, navigation and spatial memory;
- Drug countermeasures;
- Postflight locomotion and gaze assessment;
- Neurovestibular rehabilitation;
- Vestibular effects on autonomic function;
- Effects of weightlessness, stress, isolation, immobilization and diet on vestibular function; and
- Potential mechanisms for and diagnosis of irreversible neurovestibular changes.

As stated earlier, NSBRI research is ultimately directed at the development of countermeasures. Basic research projects must plausibly lead in that direction. If specific countermeasures are proposed, they will ultimately have to be proven safe and practical, and their potential impact on other physiological systems must be understood. In addition, the dependent measures used to assess countermeasure effectiveness must be defined. Individual differences in susceptibility to neurovestibular problems must be recognized. Currently, research is being conducted at the cognitive, behavioral, system, organ and cellular level, using quantitative techniques in both humans and animals. Molecular methods may also be appropriate. Much of the research is interdisciplinary and involves collaborations between investigators at multiple institutions. Use of mathematical models as a research tool is encouraged. (N. B., The current NASA postflight neurovestibular assessment is a 15-minute exam of neurological symptoms and signs, dizziness, motor performance and gait.)

Research Questions

The following questions are provided to assist the applicant in developing a proposal that is focused on relevant research. The questions are listed under the nine themes. They are not complete, and many questions are relevant to more than one theme. Project proposals may address other questions fitting within the programmatic interests defined above.

Adaptive Generalization and Context-Specific Adaptation

- Can we enhance an individual's ability to adapt to multiple environments through adaptive generalization? What are the sensory-motor responses that must change in a functionally adaptive manner during prolonged space flight? Does such adaptation does take place? How can it be reliably measured? Can these adaptive responses be trained to be context-specific?
- What is the evidence for and the physiological bases of oscillopsia, disorientation, ataxia and reduced dynamic visual acuity reported by crewmembers, particularly while making head movements during re-entry and immediately postflight?
- To what extent can gravireceptor-dependent motor responses be pre-adapted in context-specific ways, so astronauts can rapidly transition between 1-G and 0-G, 0-G and partial G, or 0-G and artificial G with minimal performance impairment or motion sickness? How long does the pre-adaptation last? Must the context cue be associated with active movement?

- How do countermeasures (e.g., artificial gravity, inflight exercise or preflight training) affect adaptation rates and levels? How do rates and levels associated with physiological (sensorimotor, autonomic, emetic) adaptation to microgravity and 3/8 G on Mars correlate with operational performance changes?
- What are the appropriate space-flight analog environments that can be used as test beds for evaluating neurological adaptation, adverse operational implications, countermeasures and impacts of adaptation on other anatomical and physiological systems?

Artificial Gravity

- What are the pros and cons of artificial gravity (AG) as a countermeasure against the effects of 0-G on neurovestibular function and on cognitive and physical performance? What are the advantages and disadvantages of large radius continuous AG vs. short radius intermittent AG, and how are these influenced by mission duration and post-landing environment (Mars vs. Earth)?
- Can humans successfully adapt to working perpendicular to the angular velocity vector?
- How can transitions be eased?
- What is the maximum tolerable rotation rate for a given G level? What is the best habituation schedule?
- What is the relationship between psychosocial factors and vestibular adaptation to altered gravity?

Visual (Multisensory) Orientation, Navigation and Spatial Memory

- How do visual and nonvisual cues interact to influence human orientation perception and perceptual motor behavior? Does 1-G training in simulated "agravic" real or virtual environments improve 3-D spatial memory and performance in orientation and navigation tasks?
- How do visual, vestibular and haptic cues contribute to inversion illusions, visual reorientation illusions, extravehicular-activity acrophobia, disorientation and poor 3-D spatial memory in 0-G?
- What is the physiological basis of inversion illusions, visual reorientation illusions, EVA acrophobia, disorientation and 3-D spatial memory problems in 0-G?
- How is the human sense of place and direction neurally coded in 0-G?
- Can preflight training techniques (e.g., virtual reality simulations) be used to alleviate these problems and to evaluate emergency procedures?
- How can 0-G immersive teleoperation displays be designed to reduce disorientation and/or motion sickness?

Drug Countermeasures

- Can improved anti-motion sickness drugs, delivery systems and dose and side effect monitoring systems be developed? Drugs must be effective, easily and safely used over days to weeks with minimal side effects and must not impair adaptation. Ground-based experimental models for evaluating 0-G pharmacokinetics and for assessing the effectiveness of drug countermeasures are needed.

Postflight Locomotion and Gaze Assessment

- What causes the profound impairments of posture, gaze and locomotion stability in many returning astronauts (and in vestibular patients), and how can these be quantified?
- What causes the large differences in level of impairment observed among different people? How do these differences correlate with physiological and operational performance changes?

- How are the multiple, mutually dependent sensorimotor systems responsible for locomotion altered by exposure to space flight? For example, what is the role of the vestibulo-ocular, vestibulo-collic and vestibulo-spinal reflexes in 3-D control of locomotion?
- How are target acquisition, smooth pursuit and saccadic mechanisms programmed during locomotion? How do oculomotor and gait control systems interact during locomotion and head turning? How is this interplay affected by space flight?
- Can long-term exposure to space flight impair sensorimotor plasticity?
- What roles do visual cues play in postflight locomotor control?
- In an altered sensory environment, does motor control require increased cognitive resources? Does this multi-tasking impair performance? Can a dual-task paradigm be used to monitor adaptation?
- What is the linkage between space-flight induced changes in sensory-motor control and astronaut functional performance?
- What measures represent composite and global indicators of locomotor and/or gaze dysfunction after space flight? What measures are the most efficient and sensitive indicators of changes in locomotion and/or gaze? What is their correlation with functional performance after space flight.

Neurovestibular Rehabilitation

- Can preflight or inflight training, sensory aids, prostheses and assessment techniques improve inflight orientation and gaze control? Can they improve postlanding functional task performance and also postural and locomotor control?
- How can somatosensory information be used to assist adaptation?
- What are the relative contributions of neurovestibular adaptation, neuromuscular deconditioning and orthostatic intolerance to postflight neuromuscular coordination, ataxia and locomotion difficulties?
- How does attention to a new sensory-motor task affect performance of a secondary task?

Vestibular Effects on Autonomic Function

- What is the physiological basis of space motion sickness? How does chronic space motion sickness (including sopite syndrome) affect mood, initiative and interpersonal relationships?
- Does the neurovestibular response to weightlessness impair postlanding cardiovascular regulation and contribute to orthostatic intolerance? In what effective frequency range? Can an effective countermeasure (e.g., AG) be developed to exploit this knowledge?

Effects of Weightlessness, Stress, Isolation, Immobilization and Diet on Vestibular Function

- How can changes in vestibular function due to weightlessness be distinguished from the normal responses to stress, isolation, diet and normal background physiological variability? What countermeasures can be developed?

Potential Mechanisms For and Diagnosis of Irreversible Neurovestibular Changes

- How might very long duration exposure to 0-G or partial G cause irreversible (pathophysiological) changes in central or peripheral vestibular function or development, or cause acceleration of the normal aging process? Would some individuals be more susceptible than others? What is the potential time course? How could such changes be reliably detected at an early stage?
- How does serum calcium homeostasis impact otoconial turnover?

3.8 Radiation Effects

Team Leader: John F. Dicello, Ph.D.
Johns Hopkins University School of Medicine
(See Table 2)

Exposure to higher than normal radiation levels is one of the major health risks to humans on long-term space flights (see the Critical Path Roadmap, Section 3.1). This exposure results primarily from galactic cosmic rays (GCR) and solar particle events. The protons and high Z, energetic particles (HZE) involved may exert sizable biological effects even at low fluence, and there are considerable uncertainties associated with secondary particle effects (e.g., HZE fragments, neutrons, etc.). Although the health risks from exposure to radiation (x rays, gamma rays, or electrons) encountered on Earth are comparatively well known, the health risks from space radiation are not well known. Several independent causes contribute to the overall risk to astronauts exposed to the complex space environment on exploration-class space missions. Of primary concern is the induction of cancer. However, central nervous system damage is also a potentially mission-compromising event because of the possibility of cell loss from radiation damage affecting central nervous system functional integrity. Recent studies also point to previously unknown mechanisms of radiation-induced cellular pathologies based on the communication between damaged and undamaged cells and the induction of unstable states that lead to late expression of genetic damage. Space radiation appears to be uniquely effective in causing such cellular changes.

The current NSBRI radiation research program is highly focused on one model system: the mammary tumor system in the female Sprague-Dawley rat. The biological endpoint being addressed is cancer. Institute resources for this research area are not likely to be sufficient in the near term to cover the full range of risk-related radiation research but must be used to address only topics of the highest priority, where substantive results and accomplishments can be expected in a single-award time frame. Therefore, research applications should propose projects with direct applicability to one or both of the following problems:

- Improving the predictions of risks to human health from space radiations, and/or
- Providing effective countermeasures that will significantly reduce these risks.

Each application must provide a strategy and schedule that would describe how the results of the proposed experiments would finally yield data that could be used directly for providing a quantitative estimate of risk or for producing an effective countermeasure. It is important that this strategy be as explicit as possible and contain a schedule that would yield results within the necessary time frame.

The countermeasures referenced here are biological or biochemical agents useful for modulation of significant radiation effects, which offer substantial promise as prevention or intervention tools in managing of human risk arising from space-radiation exposures. Proposed agents shall have demonstrated efficacy for chemoprevention of malignancies with low or no significant toxicity. Radiation countermeasure agents shall be based on scientific understanding of their likely efficacy against protons and high-energy, highly charged nuclei (HZE particles).

Research in this area will focus on five interrelated themes:

- Development of countermeasures for mitigating the effects of radiation exposure;
- Development of markers for determining risks and monitoring the efficacy of countermeasures;
- Determination of carcinogenic and CNS effects of space radiation;

- Determination of acute and long-term pathological responses of rapidly renewing organ systems at risk; and
- Characterization of differences in cell and molecular mechanisms for pathological effects for high- versus low-linear energy transfer (LET) radiation in defined model systems.

Research Questions

The following questions are provided to assist the applicant in developing a proposal that is focused on relevant research. They are not complete, and project proposals may address other questions fitting within the programmatic interests defined above.

- What is the probability of cancer induction and/or CNS damage by protons and HZE's in animals?
- Are there complementary *in-vitro* cellular and subcellular experiments that can be done to establish the mechanisms of carcinogenesis *in-vivo*?
- Are there chemical or biological agents that can be implemented to mitigate radiation risks?
- Are there radioprotectants that mitigate acute exposures?
- Are there classes of minimally toxic agents that will globally reduce radiation risks?
- Are organ-specific countermeasures useful for reducing radiation risks on long-term space missions?
- What is the methodology to extrapolate the biological results to human risk?
- How can existing epidemiological data for humans be utilized to interpret biological data in terms of risk assessments for exposures in space?
- What are methodologies to extrapolate biological results to low-dose risk predictions?
- Are there effects or mechanisms associated with high-LET interactions which are not produced in low-LET interactions?
- Are the risks from the various radiations in space independent?
- Are there environmental stressors that exacerbate the disease risks?
- Do changes in radiation quality as a function of spacecraft material and thickness significantly alter risk?
- Are the biologic effects at low doses resulting from primary, incident protons sufficiently similar to photons that the photon data can be used for proton exposures?
- What is the dependence of biological response on fluence and fluence rate?
- Are the single-particle events from the HZE's in space properly simulated with present accelerator-based exposures?
- What non-radiation factors contribute to the observed radiation responses?
- Are there significant risks from radiations in space other than those associated with carcinogenesis and CNS damage?
- Are there short-term or intermediate-term biomarkers that can be used to monitor biological consequences of radiation exposure with adequate sensitivity and lack of confounding factors?
- Are there nutritional supplements that will provide radiation protection and/or boost organ function and/or environmental factors that can significantly alter radiation risks in space?

3.9 Technology Development

Team Leader: Vincent L. Pisacane, Ph.D.
Johns Hopkins University Applied Physics Laboratory
(See Table 2)

The goal of the this research area is to develop technologies that will lead to a better understanding of the barriers to long-duration space exploration and to assist in the development of countermeasures to assure safe and productive missions. Primary attention will be directed toward technologies that support the ground-based and space-flight research of the other eleven NSBRI research areas. Seven of these areas are described in this announcement and four are described in a previous announcement (Announcement NSBRI 99-02, December 28, 1999). Such research may include studies involving either human or animal subjects, or both. The Technology Development Team will create systems and tools such as sensors, instrumentation, devices and intelligent software and systems that support the NSBRI research teams and the space life sciences research community at large. The tools developed will be used for studies including human and animal subjects, to develop countermeasures and to support remote medical care. The specific objectives of the technology development program are to develop sensors, devices, instrumentation and systems that: support the investigation of the effects of space flight on human physiology and behavior; apply this information toward developing the techniques and technologies that will sustain humans during future missions through the development of countermeasures; and benefit the quality of life and medical care on Earth. Specific technologies of interest include those that:

- Identify the risks of space flight to humans,
- Identify the impediments to effective work and operation on and near other celestial bodies,
- Assess the physiological and psychological status of test subjects,
- Determine the a proposed countermeasure's effectiveness,
- Monitor a countermeasure's effectiveness during space flight,
- Support remote health maintenance and medical care, and
- Exploit these advances to improve the quality of life on Earth.

A subsidiary interest is to promote technology transfer by collaborating with industry early in the development process, especially utilizing the NSBRI's Industry Forum, so that the products can be made available to support other research activities that can benefit society.

The Technology Development Team will generally focus on projects that will deliver a specific product in a specified period of time, typically one to three years. Proposals will be expected to be of a maturity equivalent to that of a typical NASA Phase A (Conceptual Design) study.

The following eight interrelated themes will be used to build a focused technology development program:

A. **Monitoring/Sensing Systems.** Systems are needed for monitoring humans and animals in both Earth- and space-based research protocols. A broad range of measures, including physiology, performance and environment, are needed along with methods for telemetering and storing the information.

B. **Sample Collection & Processing.** Success of many research protocols is predicated on the collection, handling and processing of biological specimens. New and unique methods are required to collect samples, with minimal impact on the subject, and to reliably process the samples in near real-time.

C. **Analysis/Decision Support Systems.** Large amounts of biochemical, histology, assay, image and signal information need to be presented and analyzed in support of the research efforts. Advanced, automated and quantitative tools are needed to support the growing analysis and decision-support requirements.
D. **Human-Machine Interfaces.** There is a growing need to integrate machines with human subjects and researchers. It is necessary to expand interfaces to all senses for monitoring and stimulation. Virtual reality displays and enhanced control modalities should be adapted for simulation and tactical use.
E. **Informatics.** It is necessary to analytically predict physical, chemical and biological system responses. The use of modeling and simulation methods can be made practical if advanced tools and techniques are developed. Integrated and searchable data archives are also necessary.
F. **Interventional Modalities.** Therapeutic and pharmacologic methods hold promise as countermeasures for microgravity effects and as treatment for other medical conditions. Development of advanced interventional resources is necessary.
G. **Cell-Based Tools.** Experiments that require the processing of cells are manually intensive and time consuming. Automated ground-based tools and space-based methods that do not rely on gravity need to be developed.
H. **Cost-/Time-Saving Devices/Methods.** There is an overarching need to identify and implement devices and methods that will facilitate lower-cost research that can reliably be completed in less time.

Research Topics

The following topics are provided to illustrate the research scope of this area. They are not complete; project proposals may address these and other topics singly or in combination.

Advanced Non-/Minimally-Invasive Physiologic Monitoring. There is a critical need to develop or adapt existing monitoring systems that can be used in human and animal research. These monitoring systems should be non- or minimally-invasive and miniature, relative to the experimental subject, and capable of remote data transmission for both ground-based and inflight environments. Monitoring parameters of interest include vital signs, core body temperature, eye motion, body fluid chemistry and hormone, endocrine and melatonin levels.

Non-Invasive Monitoring of Soft Tissue Composition and Bone Material Characteristics. Technology is desired that would be used to monitor muscle atrophy during long-term space flight. Compact, easy-to-use methods accessible to important sites in the trunk and lower extremity are preferred. Technology is also needed to monitor the possible effects of space flight or disuse on the material properties of bone, independent of anisotropy, and must be able to structural or density effects. Methods must account for bone anisotropy and must be able to measure properties in principle loading directions in accessible lower-extremity sites.

Remote Spatial Position Measuring System. A spatial positioning system which provides accurate six degree of freedom information of human body segments is required. The device should be small, portable and light weight with low/no susceptibility to spacecraft electromagnetic interference (EMI) and magnetic field influences.

Minimal-Size Animal Data Measurement and Telemetry System. Tethered measurement systems inhibit or alter responses in small animals. Tethering humans greatly limits their ability to do the kind of work they need to do during space flight missions. The measurement of

parameters from unencumbered subjects is required. The measurement system should be extremely small, have a range of >10 feet and handle multiple channels with high resolution, large dynamic range and moderate bandwidth.

Pathogen Monitoring. The need exists to be able to detect and identify pathogens, including bacteria, fungi and viruses, in air, water samples, food and human specimens. Emphasis should be given to analysis of small sample volumes, fast read-out and automated methods.

***In Vivo* Mechano-Transduction Monitoring.** New techniques to elucidate basic mechanisms of muscle and bone atrophy and mechano-signal transduction are needed. Possible approaches include mechanically-stimulated altered fluorescence, altered gene expression and proteomics.

Novel Sample Processing Strategies. New automated delivery devices are needed to increase throughput of cell culture and small animal models in ground-based radiation experiments. An automated computer-driven image analysis system is needed for histological tissue sample analysis.

Optimized Blood/Fluid Sampling. Frequent measurement of analytes in blood or other serous fluids can indicate the need for or effectiveness of countermeasures. Ideally, a non-invasive, body-worn device that can continuously collect and analyze tiny quantities of blood or serous would be the result. Current technical constraints, however, provide only intermittent samples that may need to be analyzed by a separate instrument. The immediate need is to provide an easy-to-use, non- or minimally-intrusive method of withdrawing or collecting such fluids without the problems and discomfort of frequent blood draws.

Automated Sample Handling. Biochemical assays of cellular function require multiple steps, in which cells of interest are isolated from other cells, incubated with replacement media, exposed to particular reagents and then analyzed. The techniques for these steps in sample handling intermediate between obtaining cell samples and final biochemical analysis cannot be readily performed in microgravity. There is a need for a generic means of handling samples, perhaps using solid supports, by which sequential incubations and washes of cells might be performed.

Improved Assay Techniques. There is a need to analyze samples immediately in space flight, in order to study cell surfaces and contents (flow cytometry, surface plasmon resonance), protein contents (proteomics), gene expression (gene chips), solute composition (capillary electrophoresis, mass spectroscopy), gas composition (mass spectroscopy), etc. It is essential that equipment (not limited to the examples above) be developed for these analyses. This equipment must be small in size and mass, suitable for space flight, and either fully-automated or simple to operate by minimally trained personnel.

Advanced Gait Perturbation Device. A device to generate a small perturbation to gait during free walking is required to analyze the detrimental effects of exposure to microgravity on the balance system of astronauts. The device will be used for ground-based studies and should apply consistent perturbations across multiple trials.

Enhanced Virtual Reality Environmental System. A virtual reality (VR) system could provide stimulation to correct neurovestibular problems, enhance psychological well being and monitor performance/alertness. The deliverable system's overall properties must yield technol-

ogy that is minimally obtrusive and unencumbering, such as head-mounted displays, include multiple sensory systems and significantly extend the current sate-of-the-art VR systems.

Human Biochemistry Simulator. It has been determined that many aspects of human biochemistry change during space flight. Computer models and simulations of human biochemistry (both on Earth and in microgravity) need to be developed and validated. The biochemistry simulator would be utilized as both a predictive and diagnostic instrument. The system would be portable, self-contained, have low mass and would be used in both ground-based and space-based experiments.

Minimally-Invasive Therapy. Because medical care in a space-flight environment must be performed with limited resources, technology is needed to treat critical conditions such as blunt trauma and internal bleeding. In addition, for extended-flight scenarios, minimally invasive surgical techniques that could prevent the evolution of a critical condition and restore minimal function are desired. These systems should be portable, versatile and suitable to be performed with limited expertise in an adverse environment.

4.0 APPLICATION PROCEDURES

4.1 General Instructions

Applications are to be submitted on the grant application form PHS 398 (rev. 4/98). These forms are available electronically from grants.nih.gov/grants/funding/phs398/phs398.html. If you do not have access to the Internet, you may order the forms by calling GRANTSINFO at (301) 435-0714 or sending an e-mail to grantsinfo@nih.gov. Instructions for completing the application are found in the PHS 398 application form.

DO NOT SUBMIT THIS APPLICATION TO THE NIH. INSTEAD, FOLLOW THE SUBMISSION INSTRUCTIONS BELOW. Please direct any questions that you may have concerning this application form to the NSBRI: telephone – 713-798-7412, fax – 713-798-7413.

Submit the signed, original application and twenty-five exact photocopies and twenty-five collated sets of appendix materials, in one package, to:

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
REF: NSBRI 00-01
ONE BAYLOR PLAZA, NA-425
HOUSTON, TX 77030-3498.

Applications must be received before 5:00 p.m. CDT, Friday, June 16, 2000. FAXED proposals are not acceptable, neither are electronic mail (e-mail) responses.

4.2 Special Instructions

Research Area – Each application must address one, and only one, of the eight research areas discussed in Section 3 of this announcement. Applications that impact more than one area should be directed to only one primary research area although a secondary research area may be identified on the application. Submitters are requested to identify the primary and, if appropriate, the secondary, research area in the title blank of Section 2 of the face page of the application form (*Response to Specific Request for Applications or Program Announcement*). The "Yes" and "No" boxes may be left blank.

Potential applicants may contact the Team Leaders identified in Table 2 to assist them in determining which research area is most appropriate to apply to or to discuss the timeliness or relevance of their planned research to the research areas described in this announcement. In addition, ALL countermeasure-related proposals should contain a special statement specifying the countermeasure readiness level of the proposed project (see Section 3.1 and Table 1).

Letter of Intent – To facilitate planning for the review process, investigators are requested to advise the NSBRI of plans to submit a proposal responding to this announcement by sending a non-binding letter of intent to propose by April 14, 2000 to:

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
REF: NSBRI 00-01 – Letter of Intent
ONE BAYLOR PLAZA, NA-425
HOUSTON, TX 77030-3498.

This letter should be limited to two pages or less and should contain the names and institutional addresses of all investigators and co-investigators involved in the project, a descriptive title and the primary research area for which the proposal will be intended.

Duration of Proposed Research – Proposals for ground research may be submitted for a maximum duration of three years funding, with an assumed starting date of October 1, 2000. Space-flight investigations should be proposed for a nominal duration of three years funding, with an assumed start date of April 1, 2001. As stated below, flight investigations will be selected in October 2000 for a brief definition period. Following this definition period, proposals may be declined or selected for funding and assigned to a mission. Although some flight investigations may take longer than three years to complete, investigators are requested to assume their flight studies will be completed by October 2004.

Total Annual Cost – It is expected that the average annual total (direct + indirect) cost of selected proposals will be between $200,000 and $250,000. In general, the annual total cost of a single proposal may not exceed $400,000.

Inclusion of Women and Minorities in Research Involving Human Subjects – The NSBRI has adopted the NIH Policy regarding this matter. Thus, women and members of minority groups and their subpopulations must be included in NSBRI-supported biomedical and behavioral research projects involving human subjects, unless a clear and compelling rationale and justification is provided that inclusion is inappropriate with respect to the health of the subjects or the purpose of the research.

Human Subjects and Vertebrate Animals – For proposals involving human subjects or vertebrate animals, please follow the instructions for grant application form PHS 398 (rev. 4/98). If IRB or IACUC review is pending at the time of submission, follow-up certification of IRB or IACUC approval from an official signing for the applicant organization must be sent to the National Space Biomedical Research Institute at the address listed for proposal submission. The NSBRI will forward this information to the scientific review panel administrator. For a list of information to be included in the follow-up certification, please refer back to the form PHS 398 (rev. 4/98) instruction booklet.

Space Flight Investigations – Proposals for space-flight experiments should be submitted separately from ground-based research proposals and not combined in one package. It should be assumed that flight investigations proposed in response to this announcement will be completed by October 2004, with the space-flight resources available between October 2001 and October 2004. Investigators should note that flight resources on the Space Shuttle for the next few years and during the early phase of the International Space Station are expected to be minimal, and the competition for those resources will be intense. Thus, flight proposals should represent mature studies and be based on compelling evidence from previous flight studies or appropriate ground-based research. Flight experiments normally require limited baseline or control studies on the ground, and these should be included as part of a flight experiment proposal. It should be noted that pre- and postflight studies on crewmembers, even with no inflight data collection or protocol activity, are considered flight experiments and should be proposed as such. Preparatory ground research designed to define a flight experiment should be proposed as a ground-based study.

Investigators interested in proposing flight experiments should refer to the *Space Life Sciences Flight Experiments Information Package, 1999*, issued by the International Space Life Sciences Working Group. This package is available on the World Wide Web at

peer1.idi.usra.edu/peer_review/nra/99_HEDS_03.html.

Section 5.0 of that document concerning international application forms and instructions for proposal preparation should **not** be followed; form PHS 398 should be used instead.

Special Ground Facilities – A variety of special ground research facilities, including centrifuge facilities, bed-rest facilities, etc., are available for use by investigators submitting proposals in response to this announcement. Interested investigators are referred to the *Space Life Sciences Ground Facilities Information Package, 1999*, also issued by the International Space Life Sciences Working Group and available on the World Wide Web at the same site

peer1.idi.usra.edu/peer_review/nra/99_HEDS_03.html.

The NSBRI will negotiate appropriate use of those facilities on behalf of selected investigators, but investigators must include the cost of using these facilities in their proposal.

Special Travel and Reporting Requirements – Principal investigators selected in response to this announcement will be expected to attend two, two-day research team meetings each year at a location to be determined and one annual three- to four-day general investigator workshop/retreat in the Houston, Texas area. Budgets should reflect the costs associated with these meetings and should include a statement indicating that this travel is a special requirement. Selected investigators will become part of the NSBRI's intramural research program and will be expected to provide an annual progress report. Progress is reviewed by the NSBRI's Board of Scientific Counselors. In addition, investigators will be required to provide annual project information for inclusion in NASA's *Life Sciences Program Tasks and Bibliography*.

Data Management Plan – Most data collected through NSBRI support are required to be placed in a central Institute data archive. Investigators should plan for delivering their data to the NSBRI archive and must include the cost of data archiving in their submitted proposal. If selected, a data management plan, including a list of the data products and a schedule for their delivery, must be prepared and submitted to the NSBRI. No additional costs should accompany this plan.

5.0 COMPETITIVE PROCESS

5.1 Review and Selection Process

Applications will be evaluated for scientific and technical merit and for the likelihood that the research proposed will have a significant impact on achieving the goals stated in this announcement. The initial review will be carried out by an appropriate panel of experts convened under the auspices of NSBRI's independent Board of Scientific Counselors. As part of the initial review, all applications will receive a written critique and be discussed by the panel. Only those applications deemed to have high scientific merit will be assigned a numerical score. Applicants will receive a copy of the panel's comments and score as soon as they are available. Those proposals deemed to be in the competitive range for this submission will receive a second-level review by the NSBRI scientific program directors to determine relevancy of the proposed project to the research program in the particular research area under consideration. Applicants should be aware that some meritorious proposals may not be selected for funding. Selection recommendations are prepared by NSBRI management, reviewed by the NSBRI External Advisory Council

and approved by the NSBRI Board of Directors. (N.B. The initial review group will also examine the provisions for the protection of human and animal subjects and the safety of the research environment.)

Flight proposals may be selected for a brief definition period during which it will be determined whether or not it is feasible to actually carry out the proposed investigation in space within a reasonable time and what the realistic costs of the proposed study are. Flight proposals may be declined following this definition period.

5.2 Evaluation and Award Criteria

The following criteria will be used in the evaluation:

Significance: Is the proposal responsive to the needs of the NSBRI, as expressed in this announcement? If the aims of the application are achieved, how will scientific knowledge be advanced? What will be the effect of these studies on the concepts or methods that drive this field? What is the likelihood that the proposed research will lead to new countermeasures or tests of the utility of countermeasures?

Approach: Are the conceptual framework, design, methods and analyses adequately developed, well-integrated and appropriate to the aims of the project? Does the applicant acknowledge potential problem areas and consider alternative tactics? Are there strong interdisciplinary components?

Innovation: Does the project employ novel concepts, approaches or methods? Are the aims original and innovative? Does the project challenge existing paradigms or develop new methodologies or technologies? Are novel experimental approaches considered? Do preliminary results support the new approaches?

Investigator: Are the scientists in the project, including collaborators, suitably trained for the proposed work? Is the work proposed appropriate to the experience level of the principal investigator and other researchers (if any)?

Environment: Does the scientific environment in which the work will be done contribute to the probability of success? Do the proposed experiments take advantage of available unique features or facilities or employ useful collaborative arrangements? Is there evidence of appropriate institutional support?

Selection will be based on the merit score awarded by the peer review panel, on the programmatic relevance as determined by NSBRI management, on cost, and, in the case of flight proposals, on the feasibility of actual implementation. For studies involving human subjects, the adequacy of plans to include both genders and minorities and their subgroups as appropriate for the research goals and the plans for subject recruitment and retention will be taken into account.

6.0 SCHEDULE

The following schedule is planned for the formation of new research teams by the National Space Biomedical Research Institute:

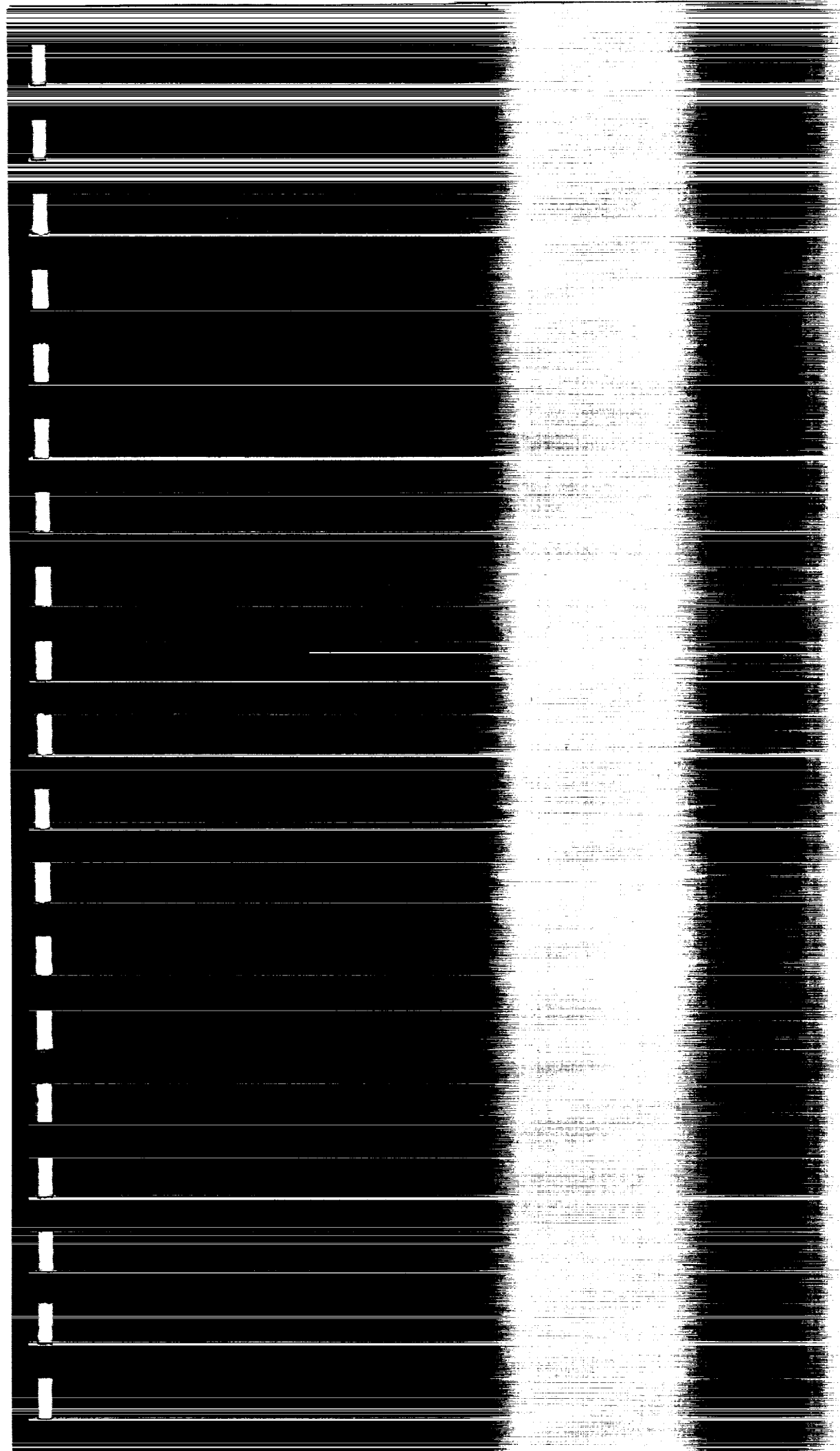
Letter of Intent Due:	April 14, 2000
Proposal Due:	June 16, 2000
Selection Announcement:	August 2000
Funding Initiation:	October 2000

Original signed by
Laurence R. Young, Sc.D.
Director
NSBRI

Original signed by
Ronald J. White, Ph.D.
Associate Director
NSBRI

Original signed by
Bobby R. Alford, M.D.
Chairman of the Board and CEO
NSBRI

February 22, 2000
Date



Appendix I

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Participants in the Eight Original Research Area Workshops

October – December 1999

National Space Biomedical Research Institute
BONE LOSS TEAM WORKSHOP
November 16-17, 1999

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National Space Biomedical Research Institute
CARDIOVASCULAR ALTERATIONS TEAM WORKSHOP
December 14-15, 1999

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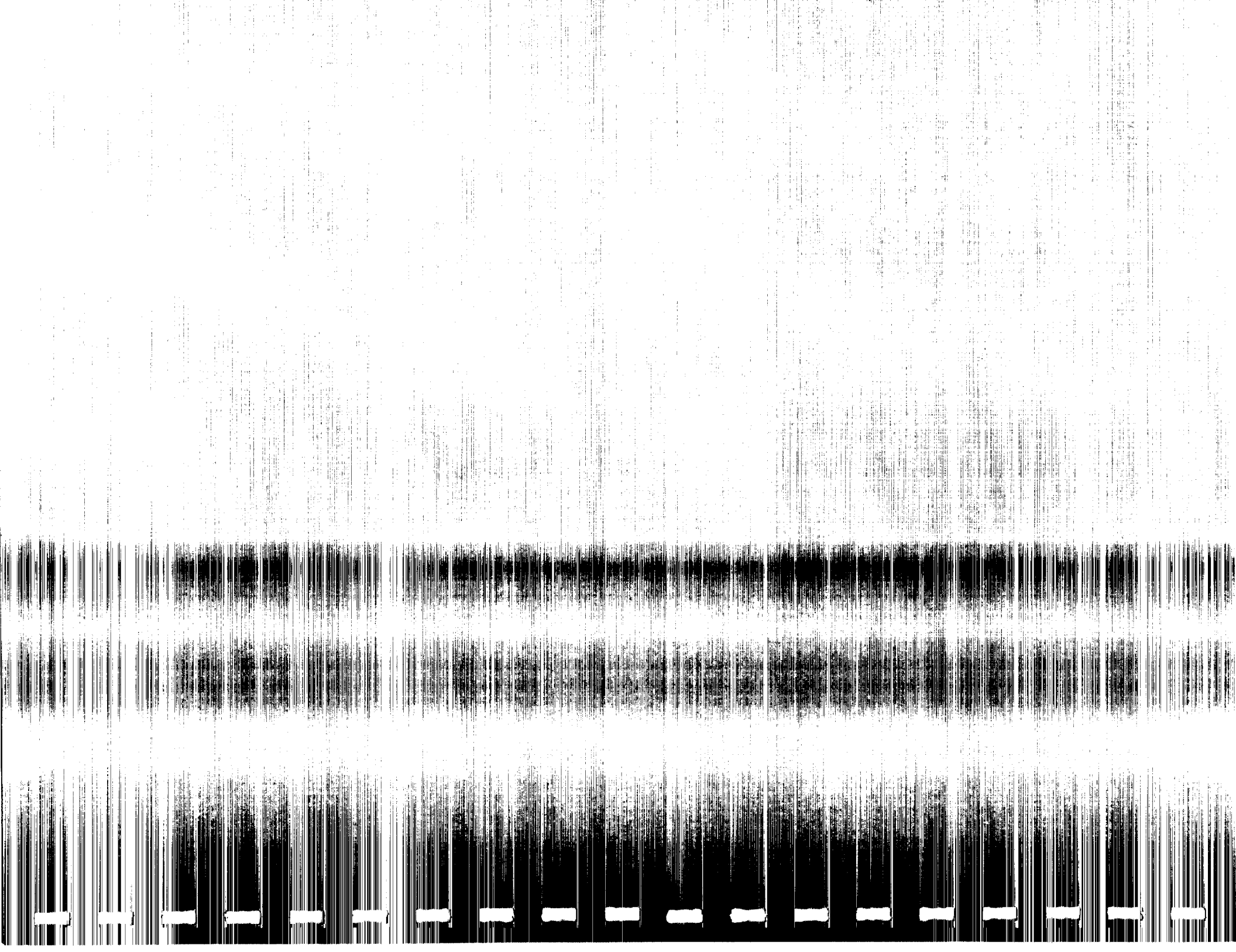
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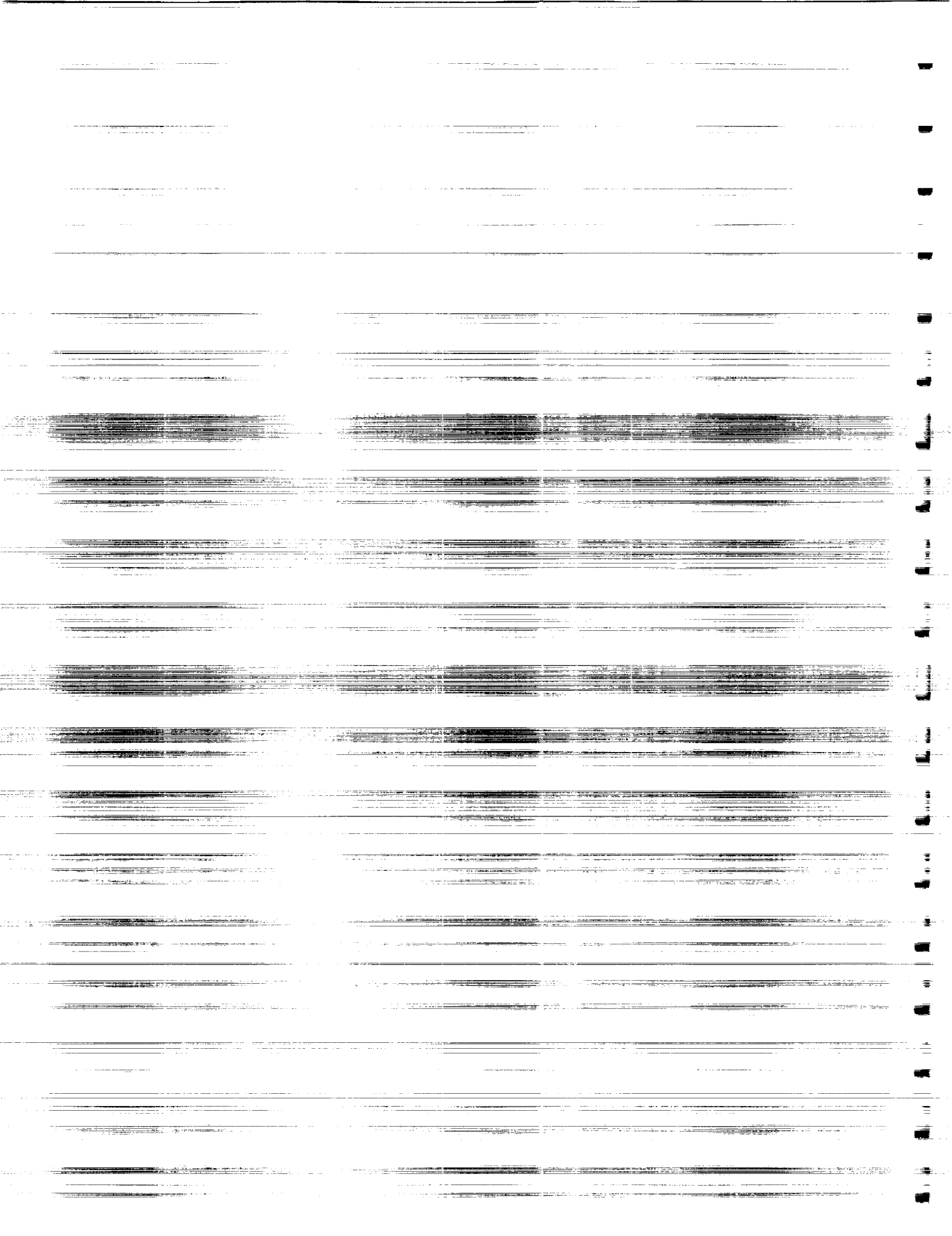
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Research Announcement 00-01
Bone Loss
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2000 NSBRI Peer Review Panel
Research Announcement 99-02
Integrated Human Function
July 26-28, 2000

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2000 NSBRI Peer Review Panel
Research Announcement 00-01
Muscle Alterations and Atrophy
August 2-4, 2000

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Research Announcement 00-01
Neurovestibular Adaptation
August 1, 2000

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Research Announcement 99-02
Nutrition, Physical Fitness and Rehabilitation
July 26-28, 2000

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Research Announcement 99-02
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Research Announcement 00-01
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August 2-4, 2000

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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE:

Guidelines for PEER REVIEW

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Appendix A: Criteria And Format For Evaluation And Scoring Of Proposals 7-9

Appendix B: Reviewer Critique Form 10-11

Note: All forms and documents can be downloaded from the NSBRI web site (http://www.nsbri.com/nsbri/nra/reviewers.cfm).

1. Review of Proposals

Proposals will be reviewed in **July and August 2000** by peer review panels of scientists with expertise in the areas of the proposals being reviewed. Additional reviews may be solicited by mail from outside experts as needed and provided to panel members for use in their evaluations. Proposals will be evaluated based on the criteria listed in Appendix A of this document. Scoring is heavily weighted toward Scientific and/or Technical merit as appropriate (70%), with relevance to the National Space Biomedical Research Institute (NSBRI) mission and goals receiving lesser weighting (30%). The appropriateness of the proposal budget will also be considered, but is not included in scoring. The results of peer review will be provided to the senior management team of the NSBRI, who will recommend selected proposals for funding based on NSBRI Programmatic considerations. Selection recommendations will then be reviewed by the NSBRI External Advisory Council and approved by the NSBRI Board of Directors.

2. Responsibilities of the Panel Chair

The Panel Chair assumes overall responsibility for the scientific review of all proposals assigned to the panel. The Panel Chair may elect to participate as a primary or secondary reviewer in the evaluation of individual proposals. The Panel Chair's responsibilities include:

- Assisting the PRA in identifying and selecting appropriate panel members;
- Assisting the PRA in determining proposal assignments to the appropriate panel reviewers;
- Chairing the peer review panel meeting and establishing the order of proposal review;
- Having a general knowledge of each proposal being evaluated and ensuring that each proposal receives a fair, impartial and complete review;
- In conjunction with the PRA, ensuring that panel discussion summaries and scores are consistent with one another and accurately reflect the consensus of the panel regarding the scientific and technical merit of each of the proposals, respectively.

In the event that an applicant appeals the result of the peer review to NSBRI, the Panel Chair may be asked to assist the PRA in reviewing and evaluating the appeal. The Panel Chair and the PRA may call upon members of the original peer review panel or other outside experts as necessary to assist in re-evaluation of the proposal.

3. Responsibilities of Panel Members

Reviewers will evaluate the scientific/technical merit of proposals assigned to the panel, as well as the relevance of proposals to the NSBRI mission and goals. The responsibilities of each panel member include:

- Serving as either primary reviewer or as one of two secondary reviewers of proposals assigned by the PRA and Panel Chair;
- Preparing independently written critiques of all assigned proposals using the evaluation criteria in Appendix A, and following the format outlined in Appendix B. **Individual**

critiques will be provided unedited to NSBRI and the applicant PI.

- **At least one week prior to the panel meeting, electronically** submitting preliminary copies of all assigned critiques to IDI (see Section 6 below);
- Bringing one (1) paper copy of each assigned critique and a backup computer diskette containing all assigned critiques to the panel meeting (see Section 6 below);
- Being prepared to discuss each assigned proposal and critique at the panel meeting;
- Introducing and beginning the panel discussion of proposals for which you are the assigned primary reviewer;
- Following its panel discussion, assigning a score to each proposal using the evaluation criteria and scoring categories described in Appendix A;
- Preparing assigned **panel discussion summaries** at the panel meeting. Each panel summary will concisely summarize the panel's discussion of a proposal (including strengths and weaknesses), and the panel's consensus regarding its scientific and technical merit. **The panel summary usually is written by the primary reviewer of a proposal**, but may be prepared by a secondary reviewer at the direction of the Panel Chair or by agreement of the panel reviewers. The assigned primary reviewer of a proposal is responsible for completion of its final panel summary regardless of the author. **The panel summary will be provided to NSBRI and to the applicant PI. A written panel discussion summary for each proposal must be completed before the panel adjourns.**

NOTE: Panel members are encouraged to read and review proposals in addition to those assigned to them for review.

4. Responsibilities of the Peer Review Administrator (PRA)

One IDI staff scientist will serve as the PRA for each panel. The PRA, with the assistance and support of other IDI personnel as needed, will manage and facilitate all aspects of the peer review process. The PRA's responsibilities are as follows:

- Explain and interpret NSBRI and IDI policies and procedures that pertain to the peer review process, including rules governing confidentiality and conflicts of interest, and procedures for panel review;
- Review proposals for responsiveness to the appropriate NSBRI Request For Proposals (RFP) and report potentially non-compliant proposals to the Program Manager for NSBRI Peer Review for further scrutiny;
- In conjunction with the Panel Chair, identify, select and recruit panel members, assign proposals to panel members for review, and identify conflicts of interest prior to, and during, the panel meeting;
- Provide copies of proposals and any other required review materials to all panel members;
- In conjunction with the Panel Chair, ensure that each proposal receives a fair, impartial and complete evaluation based on the criteria detailed in Appendix A;
- Prepare summary scores and ordered lists of proposals as required by the evaluation process;

- Assist primary reviewers and the panel chair as necessary in preparing a panel discussion summary for each proposal, ensuring that the final panel summary and score are consistent and accurately reflect the panel discussion and consensus;
- Ensure proper formatting and text editing of all final panel summaries prior to submission to NSBRI;
- Transmit intermediate and final review information to the NSBRI Senior Management Team.

5. Preparation of Preliminary Critiques

Evaluation and scoring criteria (Appendix A) are provided as a guide for the preparation of critiques and assignment of scores, and are derived from Section 5.2, p. 32 of RFP **NSBRI 00-01** (http://www.nsbri.org/) that describes the evaluation process.

Three reviewers, including one primary and two secondary, are assigned to each proposal. Each reviewer independently prepares preliminary critiques of assigned proposals prior to the panel meeting. Each preliminary critique should reflect the intrinsic scientific and/or technical merit of the proposal based only on the **Evaluation Criteria in Appendix A.** The preliminary critique should follow the format of the **"critique template" shown in Appendix B. Reviewers should appreciate that their unedited comments will be included in the official NSBRI response to the investigator.** Panel members are invited to provide written comments on any proposal under review by the panel, not just those to which they are assigned as primary reviewer or as a secondary reviewer.

Important

Preliminary critiques should be prepared and submitted electronically through IDI's secure Internet web site. *Instructions for electronic preparation and submission of critiques are included in the package of proposals and other review materials mailed to each panel member.* **A "critique template" is available on IDI's secure Internet web site. At least one week prior to the panel meeting, each reviewer should electronically submit preliminary versions of all assigned primary and secondary critiques using IDI's secure Internet web site.**

Panel members should bring one printed copy of each assigned critique to the panel meeting. It is strongly recommended that each panel member also bring to the panel meeting a virus-scanned, labeled computer diskette (3¼ inch in Macintosh or IBM-compatible format) containing backup copies of all assigned primary and secondary critiques. Files should be saved in "rich text format" (**rtf**). Reviewers should use the heading format shown on the "critique template" in Appendix A, and include the proposal number on every page of written materials.

6. Panel Meeting

The panel meeting will begin with an orientation session that will introduce the meeting participants, review procedures for the panel meeting, complete pre-meeting paperwork, and answer panelists questions. The panel will receive its charge from NSBRI senior management personnel. The panel may choose to perform a preliminary screening of the proposals at this time.

The panel will review proposals in the order determined by the Panel Chair. The Panel Chair and the PRA will ensure that each proposal receives a fair, impartial and complete evaluation based on the criteria detailed in Appendix A, and following the format outlined in Appendix B. They also will address all conflict of interest issues, and will ensure that panel members recuse themselves during discussions of proposals with which they have a conflict of interest. The PRA and Panel Chair will provide additional guidance as needed regarding peer review policies and procedures.

Proposals will be scored at the panel meeting. **Only the evaluation criteria described in Appendix A should be considered in assigning a score. The five scoring categories and their narrative descriptions are specified in Appendix A. The panel discussion summary for each proposal must be consistent with its final score.**

Evaluation of each proposal will follow the general format below:

- The primary reviewer of the proposal presents a concise summary of the proposal.
- The primary reviewer summarizes any mail reviews and scores, and then presents a concise summary of his/her critique.
- Each secondary reviewer in turn presents a brief summary of his/her critique, emphasizing areas not addressed by the other reviewers.
- Comments are solicited from other panel members followed by a general panel discussion.
- **Following the panel discussion, the primary and secondary reviewers announce their numerical scores. Each panel member records a numerical score from 0 (lowest) to 100 (highest) on the score sheets provided based on the panel discussion. The only exception to this scoring process is when a proposal is designated by unanimous decision of the panel as "Not Recommended for Further Consideration" (NRFC). In this case, each panel member must write "NRFC" on his/her score sheet for that proposal.**
- After scoring is complete, the range of scores is determined. If the range of scores does not exceed 30 points, an average score is calculated and assigned to the proposal. If the range of scores exceeds 30 points, the Panel Chair will reopen discussion of the proposal. If after additional discussion a scoring consensus cannot be reached, the reviewer whose score is outside the 30-point range will write an individual justification for that score.
- **The primary reviewer (or a secondary reviewer designated by the Panel Chair or by agreement of the panel reviewers) prepares a concise (1-2-paragraph) panel discussion summary that summarizes the panel's discussion of the proposal (including strengths and weaknesses), and the panel's consensus regarding its scientific and technical merit, as well as its relevance to the NSBRI mission and goals. The panel summary must be consistent with the proposal score.**

Important

The panel discussion summary and individual critiques of each proposal will be provided to the applicant PI and to NSBRI as written by the reviewers. IDI staff will not edit

individual critiques, and will assess each panel summary only for spelling, grammar and syntax. **The Panel Chair must approve all panel summaries and scores before the panel meeting is adjourned.** It is the responsibility of the Chair and the PRA to ensure that all panel summaries, score sheets, critiques, and other pertinent reviewer comments have been received and collected before the panel is adjourned.

7. Logistics

Travel expenses for review panel members will be paid through IDI in accordance with Government Regulations as appropriate; ground transportation costs will be reimbursed as appropriate and necessary. Other than miscellaneous personal expenses (telephone calls, personal items, etc.), panel members should expect to incur no other out-of-pocket expenses. Each panel member will be paid an honorarium for participation (except where the panel member is expressly prohibited from doing so by his/her employer), and will be required to sign non-disclosure, conflict of interest, and consultant agreements. Prior to the panel meeting, specific logistic information will be forwarded to each panel member, together with relevant review materials.

The logistics coordinator for NSBRI Peer Review is Ms. Susan Copeland (ph: 281-335-9191; e-mail: *scopeland@idinc.com*). She will oversee all travel arrangements for review panels and will be available to answer any questions regarding reimbursement procedures and honoraria. The coordinator will also make all technical arrangements related to the on-site review process and will be available to field general questions regarding computers and printers, computer configurations and any other logistic and scheduling matters.

APPENDIX A:

CRITERIA AND FORMAT FOR

EVALUATION AND SCORING OF PROPOSALS

Note: All forms and documents can be downloaded from the NSBRI web site (http://www.nsbri.com/nsbri/nra/reviewers.cfm).

EVALUATION AND AWARD CRITERIA
(from RFP NSBRI 00-01, Section 5.2, p. 32)

- **Significance:** Is the proposal responsive to the needs of the NSBRI, as expressed in this announcement? If the aims of the application are achieved, how will scientific knowledge be advanced? What will be the effect of these studies on the concepts or methods that drive this field? What is the likelihood that the proposed research will lead to new countermeasures or tests of the utility of countermeasures?

- **Approach:** Are the conceptual framework, design, methods and analyses adequately developed, well-integrated and appropriate to the aims of the project? Does the applicant acknowledge potential problem areas and consider alternative tactics? Are there strong interdisciplinary components?

- **Innovation:** Does the project employ novel concepts, approaches or methods? Are the aims original and innovative? Does the project challenge existing paradigms or develop new methodologies or technologies? Are novel experimental approaches considered? Do preliminary results support the new approaches?

- **Investigator:** Are the scientists in the project, including collaborators, suitably trained for the proposed work? Is the work proposed appropriate to the experience level of the principal investigator and other researchers (if any)?

- **Environment:** Does the scientific environment in which the work will be done contribute to the probability of success? Do the proposed experiments take advantage of available unique features or facilities or employ useful collaborative arrangements? Is there evidence of appropriate institutional support?

Selection will be based on the merit score awarded by the peer review panel, on the programmatic relevance as determined by NSBRI management, on cost, and, in the case of flight proposals, on the feasibility of actual implementation. For studies involving human subjects, the adequacy of plans to include both genders and minorities and their subgroups as appropriate for the research goals and the plans for subject recruitment and retention will be taken into account.

OVERALL PROJECT EVALUATION

I. Overall Categorization:

Each project should be placed in one of the following categories.

A. Excellent **Scoring Range: 85 - 100 Points**

Proposals in this category are superior and are definite candidates for funding as part of the research program.

B. Good **Scoring Range: 65 - 84 Points**

Proposals in this category are sound and are therefore worthy of support, but with a lower priority than those in the Excellent category.

C. Weak **Scoring Range: 0 - 64 Points**

Proposals in this category represent have significant weaknesses in one or more areas. They are not worthy of support in their present form.

D. NRFC (Not Recommended for Further Consideration)

A proposal that in the unanimous opinion of the panel is unlikely to benefit from revision *or* is not responsive to the mission and goals of the NSBRI.

E. NS (Not Scored)

A proposal that in the unanimous opinion of the panel would receive a more appropriate review in another panel will receive an NS.

II. Narrative Evaluation

The narrative evaluation for each project should reflect the strengths and weaknesses of the project in relation to the criteria listed. The score and the evaluation comments should be consistent in tone and value.

Reviewers are asked to comment on the reasonableness and appropriateness of proposed project costs in spite of the fact that this evaluation is not scored. These comments will be used in developing a final project funding plan.

APPENDIX B:

REVIEWER CRITIQUE FORM

Note: All forms and documents can be downloaded from the NSBRI web site (http://www.nsbri.com/nsbri/nra/reviewers.cfm).

Comments of the <Role> Primary Reviewer

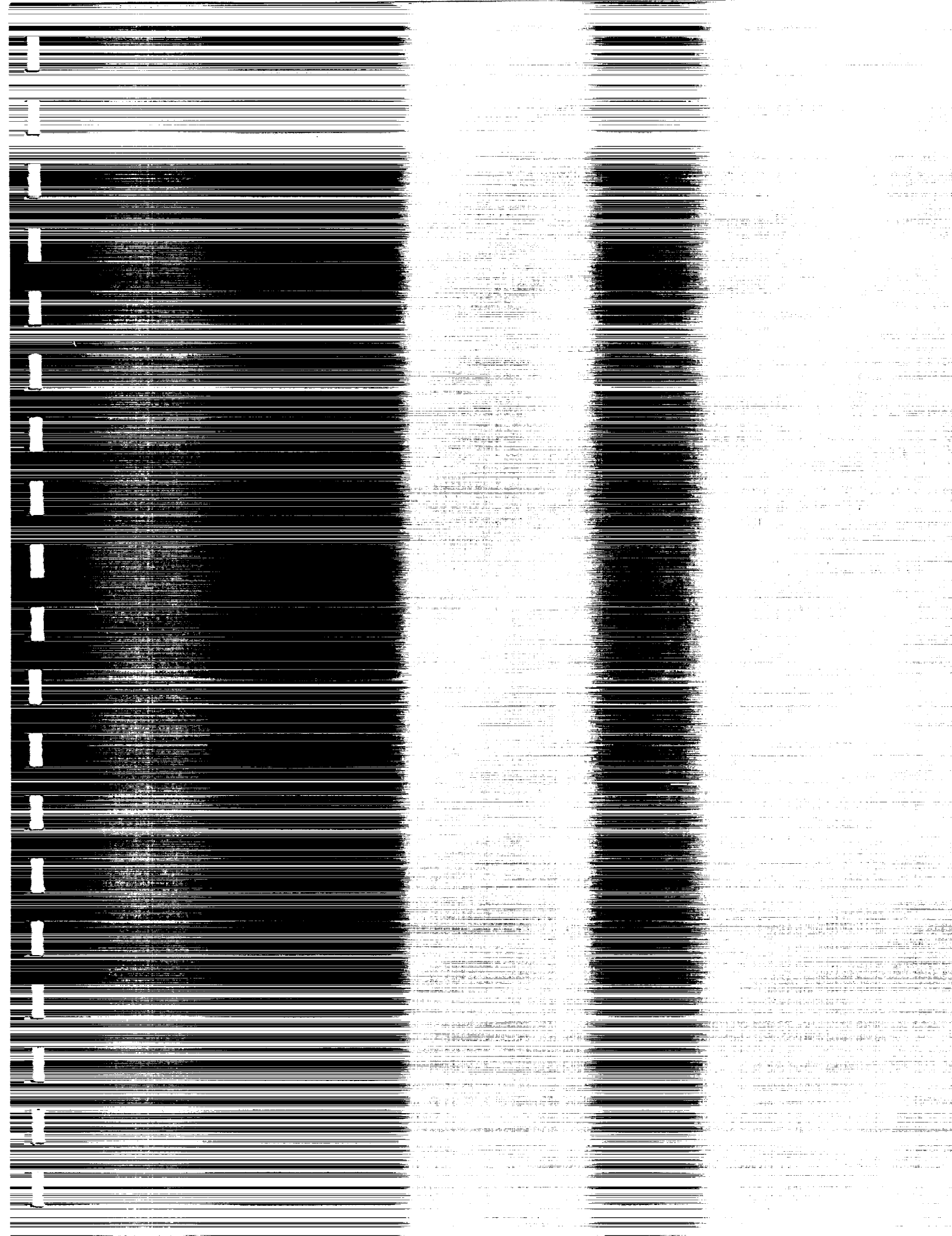
Proposal Number:	NSBRI-99-02-<###>
INVESTIGATOR:	<First Last, Suffix>
ORGANIZATION:	<Applicant Organization>
TITLE:	<Proposal Title>
PANEL:	<Panel Name>

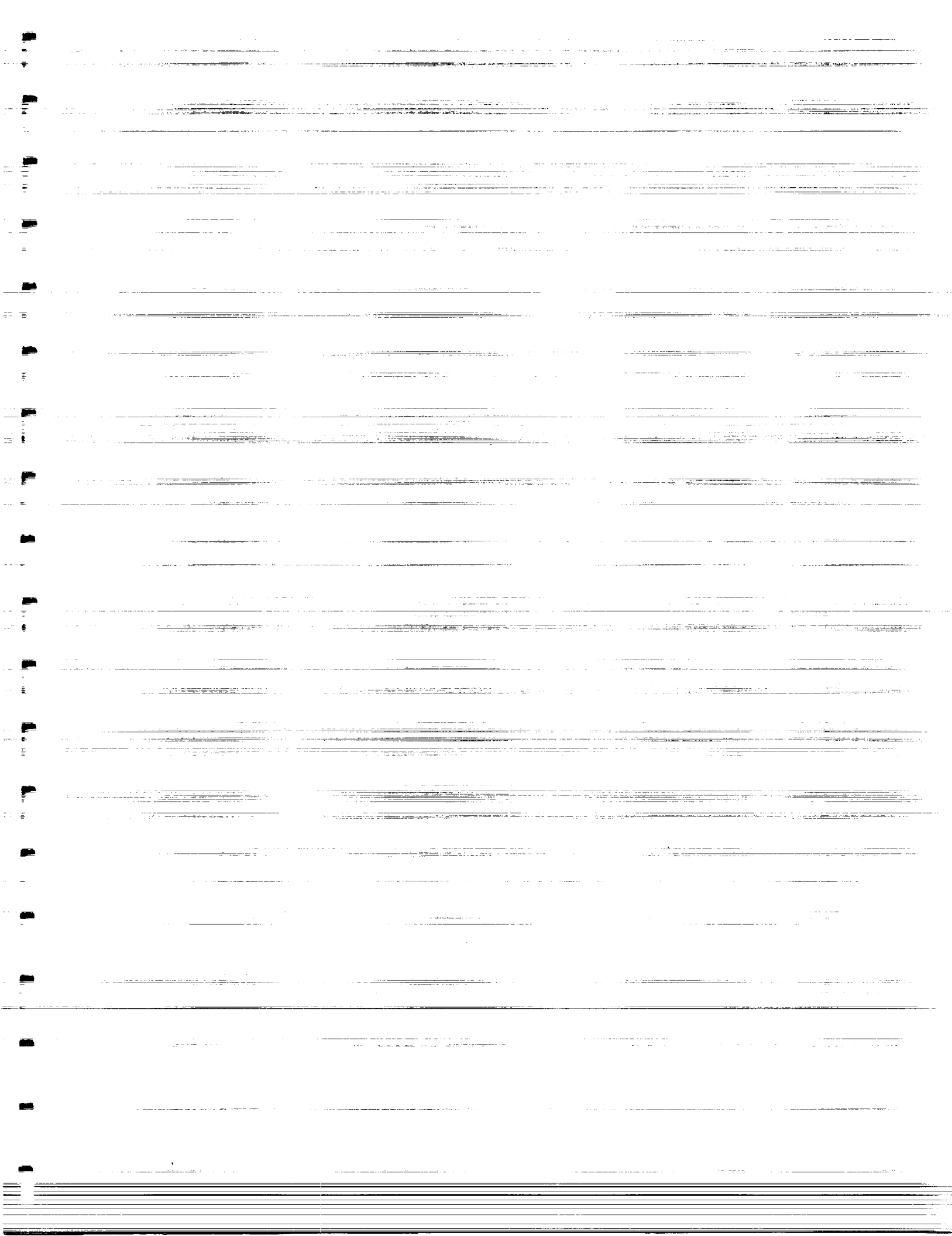
I. **Strengths**

II. **Weaknesses**

III. **Overall Evaluation**

IV. **Additional Comments Regarding Proposal Budget** (Please note that these comments are not factored into the final score)

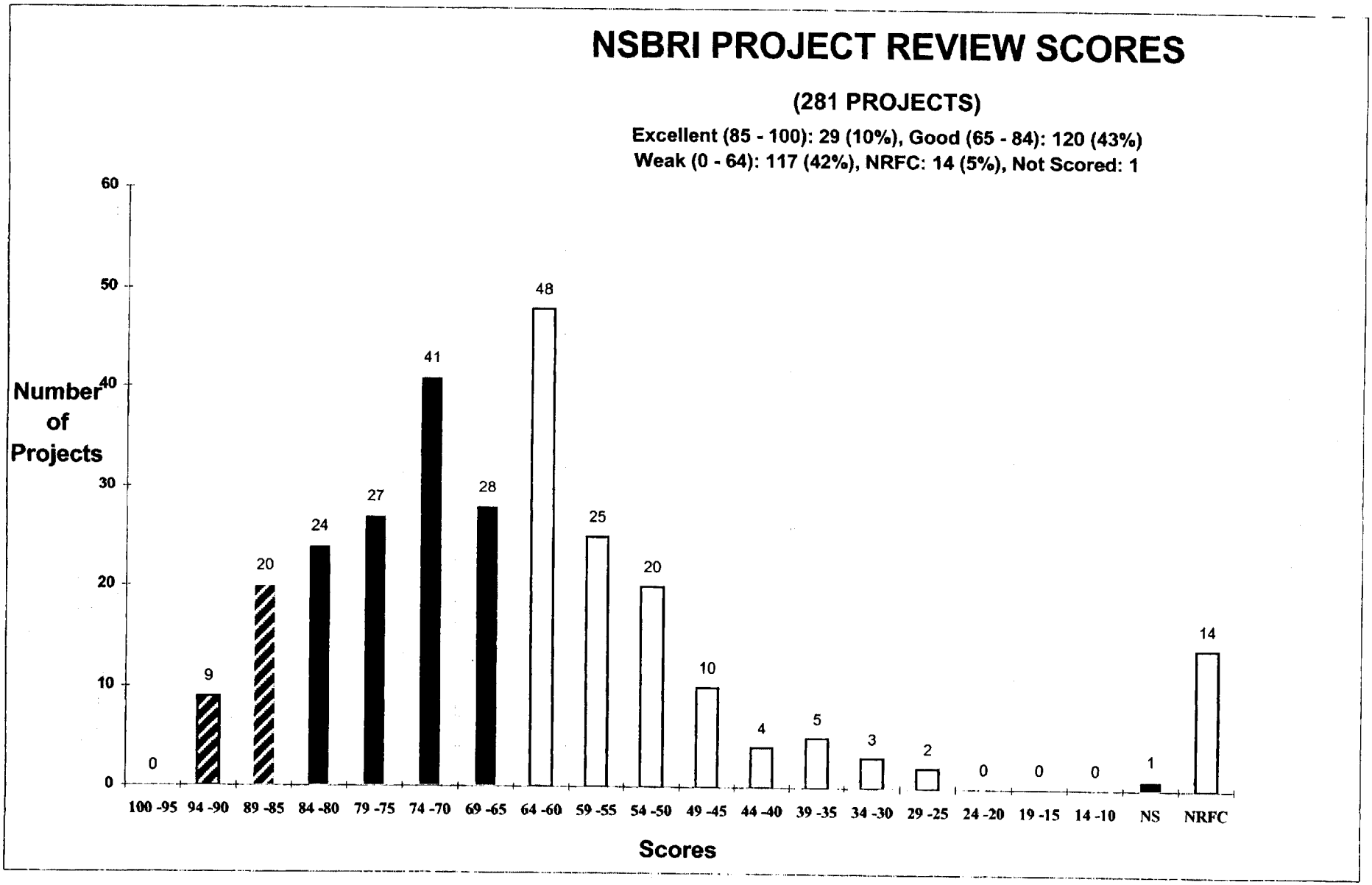


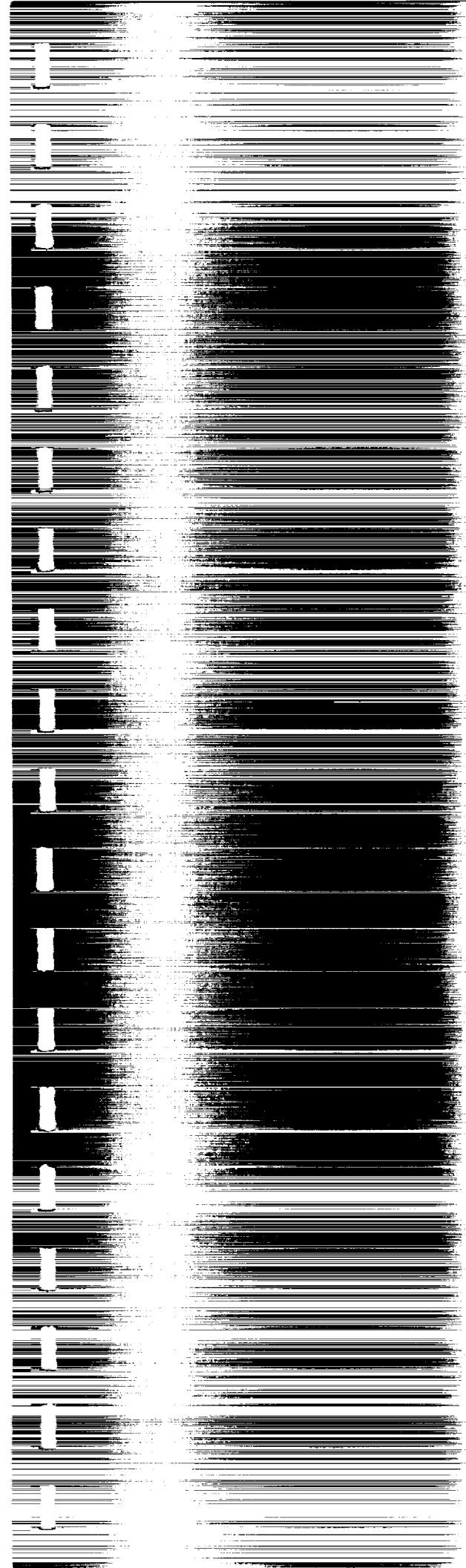


NSBRI
PROJECT REVIEW SCORES
281 PROPOSALS
(10/14/00)

Appendix K

SCORE	BONE	CARDIO	HUMAN PERF	IMMUNE	INTEGR HUMAN	MUSCLE	NEURO BEHAV	NEURO VESTIB	NUTRI, FITNESS	RADIAT	SMART MED	TECH	TOTAL
100-95													0
94-90		1		2	1	1	1	1			2		9
89-85	3	3	4	1	5	1	1	1				1	20
84-80	7	4	3			3	3	1			1	2	24
79-75	5	3	2	2	1		1	1	4	1	5	2	27
74-70	6	6	4	1	3	4	5	3	1	2	4	2	41
69-65	3	2	3	1		3	5		3	2	1	5	28
64-60	3	3	5	6	2	6	2	1	8	2	8	2	48
59-55	1		1	2	1	6	2	3	3	3	1	2	25
54-50	1	1	2	6	1	2	1		2	1	3		20
49-45				1	1	1			6		1		10
44-40				1	1				1		1		4
39-35	1			3		1							5
34-30						1	1		1				3
29-25	1			1									2
24-20													0
19-15													0
14-10													0
NS										1			1
NRFC	1	1	1	1		3	3	3	1				14
TOTAL	32	24	25	28	16	32	25	14	30	12	27	16	281


NSBRI PROJECT REVIEW SCORES
(281 PROJECTS)
Excellent (85 - 100): 29 (10%), Good (65 - 84): 120 (43%)
Weak (0 - 64): 117 (42%), NRFC: 14 (5%), Not Scored: 1
Number of Projects
60
50
40
30
20
10
0
0
9
20
24
27
41
28
48
25
20
10
4
5
3
2
0
0
0
1
14
100 -95
94 -90
89 -85
84 -80
79 -75
74 -70
69 -65
64 -60
59 -55
54 -50
49 -45
44 -40
39 -35
34 -30
29 -25
24 -20
19 -15
14 -10
NS
NRFC
Scores



National Space Biomedical Research Institute

RETREAT
January 10-13, 2000
Del Lago Conference Center
Montgomery, Texas

AGENDA

Monday, January 10

ALL PLENARY SESSIONS HELD IN TEJAS BALLROOM II & III

8:00 a.m.	Welcome Future Growth of the NSBRI	B. Alford
8:30	Expansion of the NSBRI Research Program New Research Areas & Research Announcements	L. Young
9:00	NASA's New BIOASTRONAUTICS Program	D. Williams
10:00	BREAK	
10:30	General Team Presentation: Bone Team	J. Shapiro
12:30 p.m.	LUNCH	
1:15	General Team Presentation: Muscle Team	R. Schwartz & S. Hamilton
3:15	FREE DISCUSSION TIME	
5:30	Detailed Discussion Period: Bone & Muscle Auxiliary Side Rooms: Austin Sam Houston II	Shapiro/Schwartz/Hamilton
6:30	BUFFET DINNER	
8:00	Space Flight Opportunities – OPEN DISCUSSION	C. Sawin L. Young Investigators with Space Flight Experience
10:00	ADJOURN	

Tuesday, January 11

ALL PLENARY SESSIONS HELD IN TEJAS BALLROOM II & III

8:00 a.m.	General Team Presentation: Neurovestibular Team	C. Oman
10:00	BREAK	
10:30	General Team Presentation: Cardiovascular Team	R. Cohen
12:30 p.m.	LUNCH	
1:15	General Team Presentation: Technology Team	V. Pisacane
3:15	FREE DISCUSSION TIME	
3:30	SPECIAL *OPTIONAL* WORKSHOP CRITICAL PATH DELIVERABLES	J. Charles and L. Leveton

This mini-workshop is designed to involve the NSBRI investigators in the development of specific end-items necessary to reduce the risks of space flight. A list of such deliverables and their delivery schedule has already been developed by JSC and this will be provided for discussion and criticism. *Open to interested investigators.*

5:30	Detailed Discussion Period: Neurovestibular, Cardiovascular & Technology		Oman/Cohen/Pisacane
	Auxiliary Side Rooms:	Sam Houston III Bluebonnet Montgomery III	
6:30	BUFFET DINNER		
8:00	Data Archive Project: Status & Plans		L. Suther
10:00	ADJOURN		

Wednesday, January 12

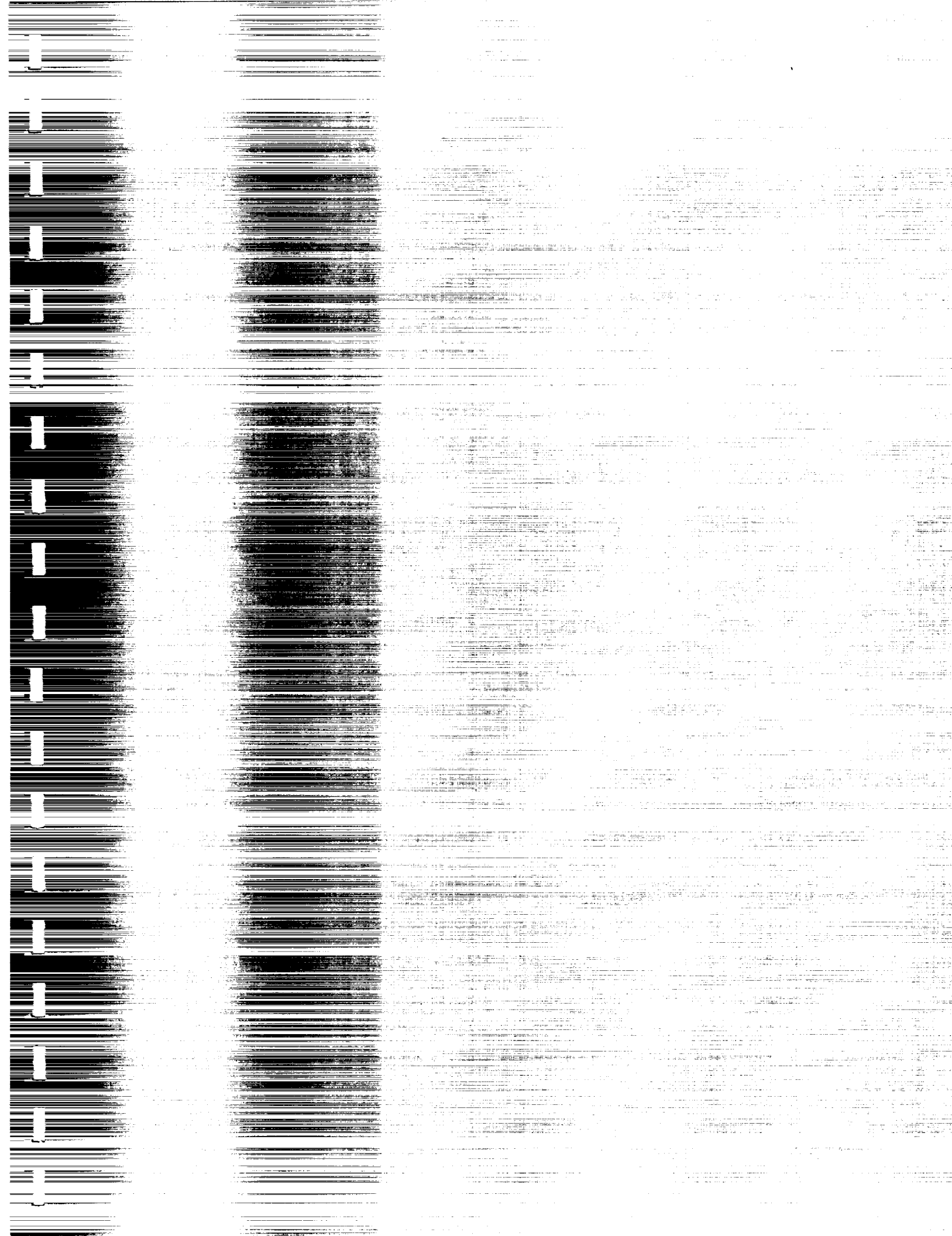
ALL PLENARY SESSIONS HELD IN TEJAS BALLROOM II & III

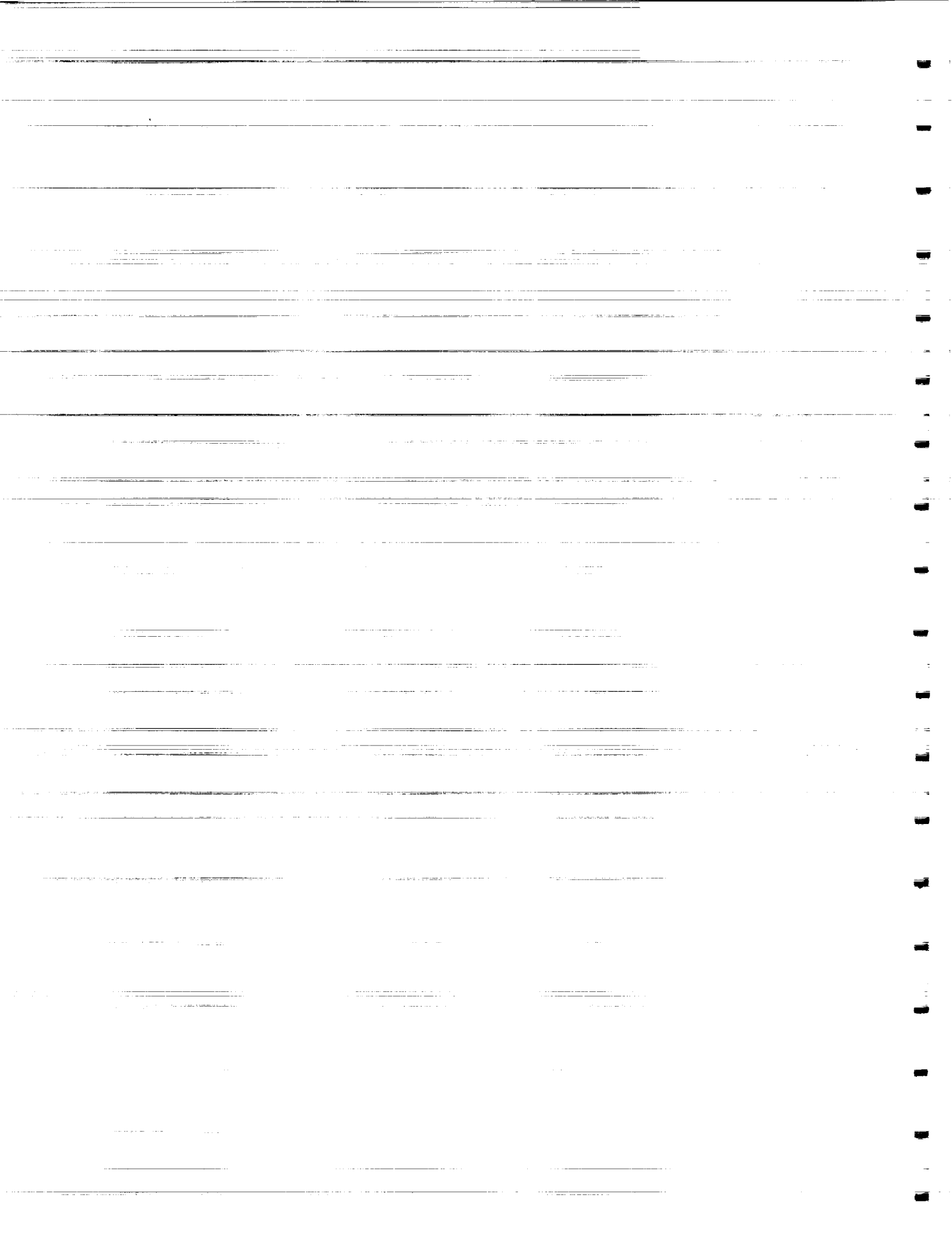
8:00 a.m.	General Team Presentation: Performance Team	C. Czeisler
10:00	BREAK	
10:30	General Team Presentation: Immunology Team	W. Shearer
12:30 p.m.	LUNCH	
1:15	General Team Presentation: Radiation Team	J. Dicello
3:15	FREE DISCUSSION TIME	
5:30	Detailed Discussion Period: Performance, Immunology & Radiation Auxiliary Side Rooms: Sam Houston IV Sam Houston I Sam Houston V	Czeisler/Shearer/Dicello
6:30	BUFFET DINNER	
8:00	Education & Outreach Program	M. MacLeish
10:00	ADJOURN	

Thursday, January 13

ALL PLENARY SESSIONS HELD IN TEJAS BALLROOM II & III

8:00 a.m.	Team Leader Reports on Workshop Results • Bone – J. Shapiro • Muscle – R. Schwartz/S. Hamilton • Neurovestibular – C. Oman • Cardiovascular – R. Cohen • Technology – V. Pisacane • Performance – C. Czeisler • Immunology – W. Shearer • Radiation – J. Dicello • Data Archive – L. Suther • Education & Outreach – M. MacLeish	
10:30	BREAK	
10:45	FINAL REMARKS	L. Young
11:15	ADJOURN	
11:30	LUNCH	





NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Special Program Announcement
for
NSBRI Consortium Institutions

An Opportunity to Participate in the

Education and Public Outreach Program of the

National Space Biomedical Research Institute

EXPANSION OF EDUCATION AND PUBLIC OUTREACH ACTIVITIES

June 19, 2000
NSBRI 00-02

Letter of Intent Due:	**August 11, 2000**
Proposals Due:	**September 15, 2000**

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Special Program Announcement
for
NSBRI Consortium Institutions

An Opportunity to Participate in the Education and Public Outreach Program of the National Space Biomedical Research Institute

Expansion of Education and Public Outreach Activities

June 19, 2000
NSBRI 00-02

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2.1 Current Education and Outreach Program 3

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3.2 Typical Program Activities 5

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TABLES

1. Current NSBRI Institutional Leaders for Education and Public Outreach 4

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Program Announcement

An Opportunity to Participate in the Education and Public Outreach Program of the National Space Biomedical Research Institute

Expansion of Education and Public Outreach Activities

June 19, 2000
NSBRI 00-02

1.0 OPPORTUNITY

The National Space Biomedical Research Institute (NSBRI), a private, non-profit organization, invites applications from its consortium institutions for the support of education and public outreach activities related to space biomedical research in general, and to the NSBRI's twelve research areas in particular:

- bone loss;
- cardiovascular alterations;
- human performance factors, sleep and chronobiology;
- immunology, infection and hematology;
- integrated human function.
- muscle alterations and atrophy;
- neurobehavioral and psychosocial factors;
- neurovestibular adaptation;
- nutrition, physical fitness and rehabilitation;
- radiation effects;
- smart medical systems; and
- technology development.

The purpose of this announcement is to solicit proposals from persons wishing to serve as members of the NSBRI's Education and Public Outreach Team. This team will pursue a coordinated program of activity focussing on the mission of communicating the significance and excitement of space life sciences to local, national and international audiences, while transferring and disseminating the biomedical knowledge gained through the NSBRI's research program from the laboratory to the classroom and home.

Applications will be accepted only from the twelve institutional members of the NSBRI's consortium: Baylor College of Medicine, Brookhaven National Laboratory, Harvard Medical School, The Johns Hopkins University School of Medicine and the Applied Physics Laboratory, Massachusetts Institute of Technology, Morehouse School of Medicine, Mount Sinai School of Medicine, Rice University, Texas A&M University, the University of Arkansas for Medical Sciences, the University of Pennsylvania Health System, and the University of Washington. However, applications may include partners from all categories of organizations, public and private, for-profit and non-profit, and eligible agencies of the Federal government. Applicants are encouraged to build collaborative relationships with other colleges and universities, schools

and school districts, museums and other interested organizations to improve student (K-16) and public understanding of space biomedical research.

The mechanism of support for these activities shall be an NSBRI subagreement with funds provided by the National Aeronautics and Space Administration (NASA) through a cooperative agreement (Cooperative Agreement NCC 9-58 with NASA's Lyndon B. Johnson Space Center). Annual renewal awards are subject to an independent, external review.

2.0 BACKGROUND

The NSBRI is responsible for the development of countermeasures against the deleterious effects of long-duration space flight and performs fundamental and applied space biomedical research directed towards this specific goal. Its mission is to lead a world-class, national effort in integrated, critical path space biomedical research that supports NASA's Human Exploration and Development of Space (HEDS) Strategic Plan by focusing on the enabling of long-term human presence in, development of, and exploration of space. This is accomplished by:

- designing, testing and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight;
- defining the molecular, cellular, organ-level, integrated responses and mechanistic relationships that ultimately determine these impediments, where such activity fosters the development of novel countermeasures;
- establishing biomedical support technologies to maximize human performance in space, reduce biomedical hazards to an acceptable level, and deliver quality medical care;
- transferring and disseminating the biomedical advances in knowledge and technology acquired through living and working in space to the general benefit of mankind, including the treatment of patients suffering from gravity- and radiation-related conditions on Earth; and
- ensuring open involvement of the scientific community, industry and the public at large in the Institute's activities and fostering a robust collaboration with NASA, particularly through NASA's Lyndon B. Johnson Space Center.

The NSBRI was established in April 1997 following competitive selection by NASA. Primary support for the NSBRI's activities is furnished by NASA through a cooperative agreement although funds to support Institute activities also come from several sources, including the institutions involved in carrying out the NSBRI's programs. The cooperative agreement award is for a five and one-half year base period, lasting until September 30, 2002, and three five-year optional extensions. Base funding was initially set at approximately $10 million annually, but has begun to increase and is currently approximately $14 million (FY 2000). NASA has notified the Institute that it would like the NSBRI to expand its activities significantly and that it hopes to provide additional funds to support planned program growth beginning in FY 2001. This solicitation is being issued in anticipation of a substantial increase in the NSBRI's core research budget beginning in October 2000, an increase that will require appropriate budgetary authorization and approval by the U.S. Congress. Prospective investigators should be aware that the implementation of the plan described in this announcement is contingent upon such favorable Congressional action.

2.1 Current Education and Outreach Program

Sharing the excitement of space research and exploration is a primary goal of the NSBRI's current Education and Public Outreach Team. Through training programs, collegiate-level courses, school curriculum materials and informational pieces, the link between space health and Earth health is transferred to teachers, students and the public at large.

Drawing on the educational expertise of Baylor College of Medicine, Morehouse School of Medicine and Texas A&M University, the team designs innovative materials aimed at promoting scientific literacy and attracting more students into science, engineering and medicine. In addition to producing print materials, the team place all guides and texts developed through this program on the NSBRI web site. Curriculum materials target elementary, high school and college students. Additional information concerning the current program may be obtained by contacting the individuals listed in Table 1.

The Education and Public Outreach Team is exploring new ways to reach the public, from placing video interviews with NSBRI researchers on the web site to developing a public radio series entitled "Biomedical Science for Space Travelers." Current projects include the following.

Elementary School Materials

- *From Outerspace to Innerspace: Learning About the Human Body* – Development and dissemination of an activity-based poster for elementary students that links NSBRI research areas to related Earth-based medical conditions.

- *Sleep and Daily Rhythms* – This represents the first in a series of educational units for grades 4-6 highlighting each of the NSBRI's research areas. The next unit will address the areas of Muscles and Bones.

Middle School/High School Materials

- *The Brain in Space: A Teacher's Guide* - This middle school/high school neuroscience teacher's guide, developed previously for NASA, has been placed on the NSBRI web site to increase its accessibility to teachers. Morehouse produced this guide during the Neurolab shuttle mission.

- *Human Physiology in Space* – This high school supplementary text, produced in 1994, has been modified slightly and placed on the NSBRI web site. The book focuses on the differences between human physiology on Earth and in space, and uses actual space laboratory experiments to guide a student's learning experience. A limited number of the original, printed Teacher's and Student's Manuals are also available through the NSBRI.

College Programs

- Human Body in Space College Course - This course, a pilot at Spelman College, gives students an appreciation of the historic science challenges of space flight, knowledge of the space environment and an understanding of the biological adaptations related to a weightless environment. It will serve as the basis for designing an NSBRI national curriculum on the human body in space.

- Summer Research Program - This program encourages women and minority-group students to pursue careers in science. Students are selected from a national applicant pool to spend a research-intensive summer doing biomedical science at Morehouse School of Medicine.

Professional Development

- Teacher Academy – This program prepares science teachers to lead their peers in implementing classroom activities that emphasize the understanding of medical issues associated with long-duration space exploration and the similarities to human disease on Earth. Academy Fellows assist with the development, implementation and field testing of new NSBRI materials in their classrooms.

Table 1. Current NSBRI Institutional Leaders for Education and Public Outreach

Morehouse School of Medicine (Lead)	**Baylor College of Medicine**
Marlene Y. MacLeish, Ed.D. NSBRI Education and Public Outreach Team Leader Morehouse School of Medicine 720 Westview Drive, SW Atlanta, GA 30310 404-756-5706 404-752-1043 FAX macleim@msm.edu	William A. Thomson, Ph.D. Professor and Head Center for Educational Outreach Baylor College of Medicine One Baylor Plaza 1709 Dryden, Ste. 545 Houston, TX 77030 713-798-8200 713-798-8201 FAX wthomson@bcm.tmc.edu
Texas A&M University George Jessup, Ph.D. Coordinator, College & Computer Educational Support Texas A&M University College of Education College Station, TX 77843-4222 409-862-2099 409-845-6129 FAX gjessup@tamu.edu	

3.0 PROGRAM NEEDS

3.1 General Information

As stated above, the NSBRI's Education and Public Outreach Team's mission is to communicate the significance and excitement of space life sciences to local, national and international audiences, while transferring and disseminating the biomedical knowledge gained through the NSBRI's research program from the laboratory to the classroom and home. Among the most important Team goals are:

- promoting excellence and innovation in America's science education system;
- attracting young people to related fields in science, engineering and medicine;
- increasing scientific literacy among teachers, students and their families, and the general public; and
- engendering public awareness and appreciation of the opportunities and benefits provided by space life sciences research and by the NSBRI's own biomedical research.

It is expected that all proposals will be clearly related to the Team's mission and will satisfy one or more of the Team's goals.

3.2 Typical Program Activities

Proposals may include a wide range of activities related to the Education and Public Outreach Team's mission and goals. Typical activities could include, but are not limited to:

- creating and disseminating supplementary space life sciences educational materials for K-12 teachers and students;
- increasing students' overall scientific literacy through development and distribution of NSBRI-related materials for reading and language arts;
- providing educational opportunities and courses for undergraduate students, and professional development for teachers and administrators; and
- producing and disseminating space life sciences educational and promotional resources for students, educators, families and the general public using a wide variety of printed and electronic media.

The following activities have been identified as important to the NSBRI and are provided to assist the applicant in developing a proposal that is focused on relevant activities. They are not complete and project proposals may include other activities fitting within the guidelines above.

Educational Materials Development

Past efforts have resulted in an array of print, audio, video and Web-based materials that have largely targeted upper elementary and secondary education. These materials focus primarily on the following research areas: cardiovascular alterations; human performance factors, sleep and chronobiology; immunology, infection and hematology; muscle alterations and atrophy; and neurovestibular adaptation. Continued development and refinement of materials developed for these areas are appropriate across all levels of public education. In addition, materials development projects focusing on the remaining seven NSBRI research areas (bone loss; integrated human function; neurobehavioral and psychosocial factors; nutrition, physical fitness and rehabilitation; radiation effects; smart medical systems; and technology development) are encouraged. Applications seeking to create educational resources—in any media, including distance

education—for early elementary, middle school, secondary, and undergraduate students are encouraged.

Educational Materials Dissemination

Projects that disseminate existing or new NSBRI educational materials are encouraged. Such projects might include scientist/educator team professional development. These teams, in turn, would give presentations to elementary and secondary school teachers, university faculty, and lay audiences.

Outreach

Plans for promoting educational outreach activities nationally and internationally are encouraged. Settings might include museums, youth clubs and Saturday science programs sponsored by local schools.

Teacher Fellowships (Scientist-Education Liaisons)

Innovative approaches are sought to develop and enhance a Teacher Fellows Program to produce teacher liaisons able to link NSBRI research scientists to the Education and Public Outreach Team. Applications that prepare teacher fellows to support the development, testing and dissemination of new and existing NSBRI educational materials are appropriate.

Teacher Professional Development

Projects that strengthen or extend current NSBRI activities related to teacher professional development, or that propose new and innovative approaches, are encouraged. All such projects should recruit and prepare teachers to utilize space life science materials and foster public awareness of NSBRI scientific and technological developments.

4.0 APPLICATION PROCEDURES

4.1 General Instructions

A complete proposal will consist of the following material, in the order listed.

1. Title Page (project name, sponsoring institution, project director with contact information, and other participants with institutional affiliation);

2. Table of Contents;

3. Abstract (need(s) addressed, project goals/objectives, proposed activities, nature of partnerships involved, intended outcomes and evaluation plan);

4 Budget Information:
   - 4.1 Detailed Budget - Year 1;
   - 4.2 Detailed Budget - Entire Project Period (up to three years) Budgets should reflect realistic costs of performing proposed work.

5. Narrative:
   - 5.1 Background (including needs to be addressed);
   - 5.2 Objectives;
   - 5.3 Methodology (including plan for dissemination, if applicable);
   - 5.4 Evaluation Plan.

6. Appendix A: Biographical Information (Project Director followed by other participants, using NIH two-page CVs);

7. Appendix B, C, ...: Any Other Supplementary Information.

Use 12-point font and one-inch margins all around, on all pages. The abstract is limited to two pages, double-spaced. The narrative is limited to 15 pages, single-spaced.

Submit the signed, original application and twenty-five exact photocopies in one package, to:

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
REF: NSBRI 00-02
ONE BAYLOR PLAZA, NA-425
HOUSTON, TX 77030-3498.

Applications must be received before 5:00 p.m. CDT, Friday, September 15, 2000. FAXED proposals are not acceptable, neither are electronic mail (e-mail) responses.

4.2 Special Instructions

Letter of Intent – To facilitate planning for the review process, potential project directors are requested to advise the NSBRI of plans to submit a proposal responding to this announcement by sending a non-binding letter of intent to propose by August 11, 2000 to:

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
REF: NSBRI 00-02 – Letter of Intent
ONE BAYLOR PLAZA, NA-425
HOUSTON, TX 77030-3498.

This letter should be limited to two pages or less and should contain the names and institutional addresses of the project director and all participants involved in the project, a descriptive title, and a brief abstract of the proposed project.

Partnerships – Applicants are encouraged to describe in their proposals existing partnerships with state and federal agencies, schools, school districts and/or undergraduate institutions that will enhance dissemination of the NSBRI materials and provide additional special opportunities for strengthening the Education and Public Outreach Team. Such partnerships will be especially valuable if they will assist in promoting use of NSBRI educational materials and professional development programs on a national level.

Institutional Commitment – Applicants are expected to demonstrate strong institutional commitment through direct and in-kind contributions (personnel time commitments, materials, space, resources, etc.). It is anticipated that funding requested covers only a portion of actual project

costs, and that applying institutions will cover the remaining costs. A minimum institutional commitment amounts to 10% of the NSBRI funding requested.

Duration of Proposed Project – Proposals may be submitted for a maximum duration of three years funding, with an assumed starting date of November 1, 2000.

Total Annual Cost – It is expected that the average annual total (direct + indirect) cost of selected projects will be between $100,000 and $150,000. In general, the annual total cost of a single project may not exceed $300,000.

Special Travel and Reporting Requirements – Project directors selected in response to this announcement will be expected to attend two, two-day team meetings each year at a location to be determined and one annual three- to four-day general investigator workshop/retreat in the Houston, Texas area. Budgets should reflect the costs associated with these meetings. Project directors will be expected to provide an annual progress report.

5.0 COMPETITIVE PROCESS

5.1 Review and Selection Process

Applications will be evaluated for merit according to the criteria below and for the likelihood that the proposed project will have a significant impact on achieving the goals stated in this announcement. An initial review will be carried out by an appropriate panel of experts convened under the auspices of NSBRI's independent Board of Scientific Counselors. As part of this review, all applications will receive a written critique and be discussed by the panel. Only those applications deemed to have high merit will be assigned a numerical score. Applicants will receive a copy of the panel's comments and score as soon as they are available. Those proposals deemed to be in the competitive range will receive a second-level review by NSBRI's management to determine relevancy of the proposed project to the NSBRI's education and outreach mission. Applicants should be aware that some meritorious proposals may not be selected for funding. Selection will be based on merit, relevancy, availability of funds, balance among the various types of programs and populations served, and geographical distribution. Consideration will be given to reaching underrepresented groups, including women and minorities.

5.2 Evaluation and Award Criteria

Applications will be reviewed for completeness, clarity and responsiveness to this announcement. In addition, each application will be reviewed based upon the following specific criteria.

1. Significance
   - The likelihood that the proposed project will result in systemic change or improvement.
   - The likelihood of increasing student interest in the space life sciences through development and dissemination of novel and current materials.
   - The importance or magnitude of the results or outcomes likely to be attained by the proposed project, especially improvements in science instruction and student achievement in science.
   - The extent to which the proposed project involves the development or demonstration of promising new strategies or materials.

2. Quality of the Project Design
   - The extent to which the goals, objectives and outcomes to be achieved by the proposed project are clearly specified and measurable.
   - The extent to which the project is part of a comprehensive effort to foster and communicate the research findings of the NSBRI into educational and outreach products and activities.
   - The extent to which the proposed activities constitute a coherent, sustained program to further the goals of the Education and Outreach Team.

3. Adequacy of Resources
   - The adequacy of support, including facilities, equipment, supplies and other resources, from the lead organization and the consortium.
   - The relevance and demonstrated commitment of each partner in the proposed project to the implementation and success of the project.
   - The extent to which the costs are reasonable in relation to the objectives, design, and potential significance of the proposed project.

4. Quality of the Management Plan
   - The adequacy of the management plan to achieve the objectives of the proposed project on time and within budget, including clearly defined responsibilities, timelines, and benchmarks for accomplishing project tasks.
   - The narrative should provide a clear description of who will do what, when, where, why and with what anticipated results. The management plan should include the percentage of staff time and this should be reflected in both the budget and narrative sections.

5. Quality of the Project Evaluation Plan
   - The extent to which the methods of evaluation are thorough, feasible, and appropriate for the goals, objectives, and outcomes of the proposed project.
   - The extent to which the methods of evaluation include the use of objective performance measures that are clearly related to the intended outcomes of the project and will produce quantitative and qualitative data.

6. Quality of the Project Personnel
   - Qualifications including education and experience of key personnel.
   - Previous experience working with teachers, materials development or in formal science settings.

6.0 SCHEDULE

The following schedule is planned for the formation of new research teams by the National Space Biomedical Research Institute:

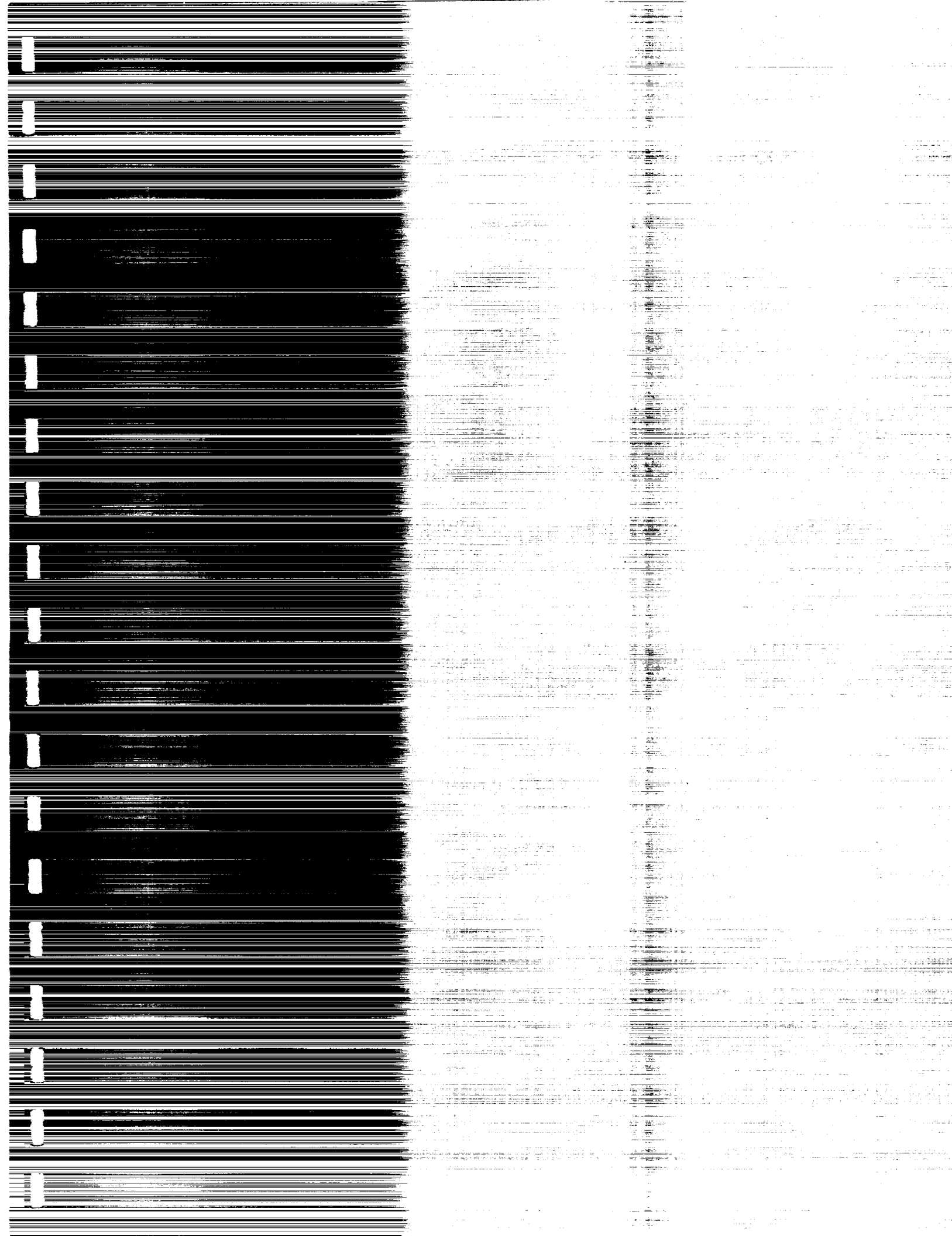
Letter of Intent Due:	August 11, 2000
Proposal Due:	September 15, 2000
Selection Announcement:	October 2000
Funding Initiation:	November 2000

Original signed by
Laurence R. Young, Sc.D.
Director
NSBRI

Original signed by
Ronald J. White, Ph.D.
Associate Director
NSBRI

Original signed by
Bobby R. Alford, M.D.
Chairman of the Board and CEO
NSBRI

June xx, 2000
Date



MULTICULTURAL AND INTERNATIONAL ISSUES IN SPACE FLIGHT RESEARCH AND HEALTH CARE

REPORT OF THE NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE WORKSHOP

Held at the
Center for Advanced Studies in Space
Houston, Texas
June 13–15, 2000

NO APPENDICES

July 28, 2000

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Executive Summary

This report summarizes the deliberations and recommendations of a working group that met in Houston on June 13th to 15th, 2000 to consider the impact of multi-culturalism and multi-nationalism on future space operations. The charge for this workshop included a request to identify the means by which to accelerate ground and space research and to identify measurements to be included in upcoming missions. Because there is a dearth of hard data on cultural issues in space flight, the broad response to the charge is to recommend elevation of psychological and cultural research to the prominence of other fields of inquiry in the scientific agenda for long duration space flight. A regimen of scientific investigation complementary to other lines of investigation into psychological and cultural factors can identify and validate operational strategies, including crew composition, mission management approaches, and countermeasures against mission stressors. General recommendations are provided including overall research approach, appropriate subject populations and research venues, methodological issues in cross-cultural research, and collaborative strategies. Specific recommendations deal with individual, crew, and organizational factors and psychiatric issues in multinational missions.

Introduction

This report summarizes the deliberations of a working group assembled to focus on multicultural and international issues that may impact the performance and psychological well-being of humans in space. The group met in Houston June 13 – 15, 2000.

The report discusses the state of current knowledge, research issues identified, and makes specific recommendations for research initiatives. Participants in the working group are listed below, with organizational affiliation, mail and email addresses provided in Appendix A. The charge to the working group by the National Space Biomedical Research Institute follows as a preface to the substance of the report.

Participants

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The University of Texas at Austin

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The Chinese University of Hong Kong

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James R. Kass, Ph.D.
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Workshop Charge

Focusing on multicultural and international issues in space flight research and crew health care, panelists will review current NASA data and information related to human space flight and the established or proposed medical countermeasures for maintaining in-flight and post-flight health. Participants also will review operational systems and research related to in-flight health care delivery and post-flight rehabilitation, the existing and proposed human-machine interface standards, and those training and operations requirements relevant to crew health, well-being and safety. This information is critical since the NASA Human Exploration and Development of Space Program will ultimately support crews that are diverse, multicultural, international, and mixed in gender and age. To reach the goal of routine space travel, issues of health, safety and performance of diverse crews must be addressed.

Following the review and discussion, the panel will develop a report with recommendations that address the following:

- The adequacy of existing demographic and epidemiological information;
- Areas needing further research;
- A method to accelerate ground and space research to ensure health, maintain crew performance and provide the best medical care to diverse crews;
- Additional measurements to be included on upcoming missions; and
- Additional clinical or scientific measurements or data to include in NASA's Integrated Test Regimen, which is currently under development.

Overview

The stimulus for the workshop was the recognition by NASA management as well as those directly involved in space missions that cultural differences have implications for the functioning of crews during space flight and the management of future missions. Recent experiences in the Shuttle-Mir program, along with concerns regarding the upcoming missions aboard the International Space Station are the most immediately identifiable reasons for this increased awareness.

What is Culture?

Scientists from different disciplines such as anthropology, psychology, and sociology have defined culture in different ways. In anthropology, culture is defined as the values and beliefs that people use to interpret experience and generate behavior, and which are reflected in their behavior. For purposes of this discussion we will use the definition of Rohner (1984) who defines culture as 'an organized system of meanings that members of the culture attribute to the persons and objects that make up the culture.' More colloquially, Hofstede (1990) has described culture as 'the software of the mind.' Smith and Bond (1999), emphasizing its subjective, interpretive quality, point out that the notion of culture should be restricted to what things mean to their members.

Three Cultures not One

Although many think of national differences as the essence of culture, in fact astronauts and cosmonauts, scientists, flight directors, and space program managers are all members of at least three different cultures – national, organizational, and professional (Helmreich & Merritt, 1998). In the case of organizational cultures, we refer to the shared values and behaviors of members of an organization such as NASA or Rosaviakosmos, or a sub-unit of an organization such as the Johnson Space Center in Houston or Star City in Moscow. Professional culture refers to the shared values and behaviors associated with a particular occupation or specialty. Astronauts and cosmonauts typically are part of several professional cultures. They are, of course, members of the elite group of spacefaring humans. At the same time they have a disciplinary specialty as pilots, engineers, physicians, or members of a scientific group such as astrophysicists, biologists, or chemists.

As is clear in its charge, the concerns of the workshop were not with culture per se, but rather with cultures as they contribute to the context in which space flight occurs and is managed. The operational focus of the workshop was to specify research needed to understand cultural influences on behavior, with the ultimate goal of assisting management in constituting missions and astronauts and cosmonauts in fulfilling the dual goals of successful task accomplishment and maintenance of their psychological well-being. Figure 1 on the following page shows the inputs, processes and outcomes associated with a mission. The three cultures constitute part of the inputs that influence processes and

outcomes and are, in turn, influenced by them for future interactions. As the figure implies, we must consider culture as part of the broader issues of individual, group, and organizational functioning.

Composition of the Workshop

To meet its charge, experts from diverse backgrounds were brought together. The membership included astronauts with first hand experience on multicultural missions, members of American, Canadian, European, Japanese and Russian national space agencies, cross-cultural researchers, and representatives of the aviation community including an airline and the International Civil Aviation Organization having first hand experience with organizational culture change and the management of multi-cultural organizations. The scientific disciplines represented included anthropology, medicine, psychology, and psychiatry.

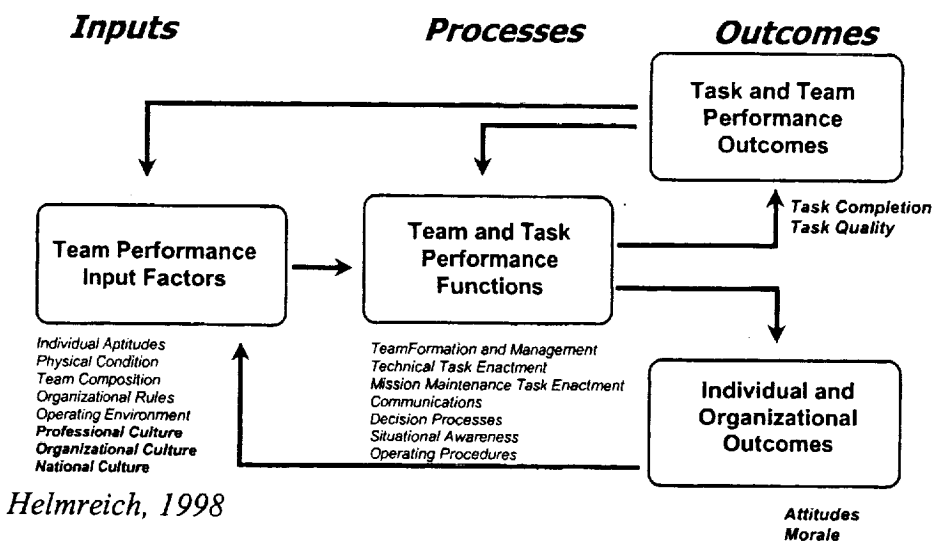


Figure 1. A recursive model of crew performance showing cultures as input factors

Formal Presentations

Members of the workshop with particular cultural experience were asked to make presentations to provide a factual and conceptual overview for deliberations. In this section these presentations are briefly summarized; the complete presentations are found in a supporting document containing appendices A through H.

Michael Bond

The first presentation was given by Professor Michael Bond of the Chinese University of Hong Kong. Professor Bond emphasized the practical utility of culture. Culture is the shared beliefs, values, expectations and behaviors that develop over time to address life's challenges. Culture enhances communication and co-ordination because it decreases uncertainty and its accompanying anxiety, thereby leading to an increased understanding of behavior, and better predictive ability (knowing what other members of the same culture is likely to do, what they are likely to say, and what it is likely to mean). The very quality of shared meanings and expectations that allow members of the same culture to interact smoothly and efficiently becomes an obstacle when interacting with members of other cultures. What was previously implicit and efficient 'short-hand' must now be detailed to avoid misunderstanding. All cross-cultural encounters are initially less efficient (more time-consuming) because of this need to explicate the rationale behind actions.

Professor Bond identified two issues relating to national culture in particular that have potential for cross-cultural misunderstanding. The first is authority ranking – the way in which leadership functions are enacted. The second is communication style and what he called levels of verbal engagement (here he contrasted the Italian and Japanese styles). The negative attributions that arise from these two issues are likely to be feelings of disrespect (challenges to the hierarchy) and/or impoliteness (disrupting socio-emotive bonds). The result can be either counter-attack or dissociation.

Professor Bond pointed out that multi-cultural teams need to experience common socialization processes that result in the sharing of perspectives and the development of a new team culture. He stressed that multicultural teams also have a requirement to address issues of first versus second language communication, the hierarchy imposed and whether it is congruent with the "home" culture, and the degree of connectedness that members have to their home culture. Missions also need to address the issue of how members of multi-national teams are evaluated by their home cultures and how tasks are allocated and shared.

Robert Helmreich

Cross-cultural research in the aviation and medical domains was reported by Professor Robert Helmreich of the University of Texas at Austin. The discussion addressed issues of performance in an environment where teams interact with technology. Areas of particular operational significance include differential acceptance of the importance of adherence to rules and power relationships between leaders and subordinates. Research in aviation has found highly significant national differences is perceptions of the need to operate in accordance with Standard Operating Procedures (SOPs). The presentation stressed the complex interplay among national, organizational, and professional cultures.

The professional cultures of pilots and doctors, as measured by surveys in multiple countries, were discussed. Both groups have strong cultures that reflect a positive desire to achieve and perform well. Both also share unrealistic attitudes regarding personal invulnerability and denial of the effects of stressors. These patterns of cultural values may

generalize to space crews and could have implications for the safety and effectiveness of flight.

The results of a survey of pilots flying in multi-national organizations were described. Respondents listed both rewards, such as challenge and learning new approaches, and a number of frustrations, particularly problems of language and communication, authority relationships, and unwillingness to admit mistakes that threaten a sense of 'face'. A methodological approach using confidential, systematic observation of flight crew behavior in normal operations was described. This research has lead to the development of a model of error and threat management in complex operations. Experience in other complex domains suggest that this methodology may be of some value in studying human performance in space. Disentangling the relationships among national, organizational, and professional cultures is essential for gaining insight into problems within the space environment and to the eventual mitigation of those problems.

Vadim Gushin

Dr Vadim Gushin of the Institute for Biomedical Problems in Moscow reported on the individual and group psychological effects observed during mission simulations. Observed reactions included depression and measurable changes in the EEG. Groups in isolation become more cohesive, but some individuals may become psychologically distanced from the group - a process he described as "alienation". Some isolated groups also show a tendency to become more self-centered and critical of external control agents, isolating themselves from 'Earth' - this process he termed "psychological closing" or "automisation".

Specifically, Dr. Gushin went on to describe in some detail the recent *Simulation of Flight of International Crew on Space Station* (SFINCSS) study that was recently conducted in Moscow. This project was characterized by both international subjects and international investigators. Complications that arose during this simulation were cited as issues that need to be addressed in future international simulations and space missions.

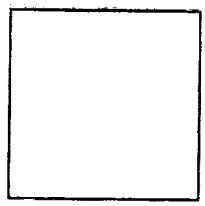
Dr Gushin also discussed specific psychological problems that have been observed in international crews during long-duration missions in the MIR space station. These include different (conflicting) hierarchies of values regarding individual and crew goals, different understanding of role distributions among crews, especially between 'guests' and 'hosts', preferences for 'own' versus host' programs, food, communications, etc, communication styles, and language problems that create emotional barriers during recreational time. As has been observed in ground simulations, Russian long duration missions have also experienced the phenomena of alienation and psychological closing. He stressed the importance of psychological preparation for multi-cultural missions through discussion, 'survival training', mutual discussion of procedures and their allocation to individuals, and group psychological training.

Daniel Maurino

Captain Daniel Maurino of the International Civil Aviation Organization (ICAO) provided a first hand perspective on the functioning of a multinational organization. Functioning as a subunit of the United Nations, ICAO is responsible for the regulation of commercial aviation in 185 countries. He demonstrated the critical role that cultural values play in determining the nature of the necessary compromises that must be made between production and safety values. In a multi-cultural agency, culture is part of the operational context and successful functioning requires that cultural values be respected rather than challenged.

Cross-cultural research in aviation has generated considerable knowledge about teamwork and team training, some understanding of safety cultures nested within national cultures, but little or no insights into cultural factors associated with technology design. The reality in aviation is that Anglo Saxon culture is the dominant culture; it designs and supplies the practices that the rest of the world uses – a reality that has both positive and negative aspects.

Citing the work of Westrum (1988), Captain Maurino characterized organizational cultures as being *Pathological, Bureaucratic, or Generative* based on their reaction to new information, their response to messengers bearing negative information, their reaction to failure, and openness to new ideas. In this framework, pathological organizations are conflicted and bureaucratic ones are buried in red tape, while generative organizations achieve high reliability. The diagram below outlines the three organizational responses. The operational impact of organizational cultures in aviation clearly translates to space flight.



Captain Maurino concluded by stating that in order to build a safety culture on the ISS, or an expedition culture for Mars, it is necessary to define the desired behaviors and let the culture be built from these behaviors. It is the organization's responsibility to define the acceptable standards for behavior.

Bruce Tesmer

Captain Bruce Tesmer of Continental Airlines built on the discussion of organizational cultures, discussing the inevitability of organizational change and the sources of change – trauma such as major disasters and proactive change through people and leadership. He presented the case study of an organization that had reached the depths through bankruptcies, mergers, and callous, rapidly changing leadership followed by a renewal

based on a strong, people-oriented leader, the acquisition and use of operational data, and open communication – in sum, the prototypical generative organization.

Leena Tomi

Leena Tomi of the Canadian Space Agency provided the perspective of anthropology on culture. Contemporary anthropology emphasizes the complexity, ambiguity, and changing nature of cultural differences, considering them social phenomena. National characteristics are dependent on the relationship between ethnic or national groups and on historical events, are vulnerable to ideological influences and are mutable at the individual and group level. She also stressed that anthropologists recognize the central role of language in reflecting cultural assumptions about other peoples and of the world around them.

Points to consider in developing cross-cultural training programs were also outlined. This included a critique of cross-cultural training materials that tend to categorize other cultures, specifically by reifying stereotypes of the culture, denying its co-evality, and presenting the trainer's culture as the universal standard. It was pointed out that the text currently used for training U.S. astronauts regarding Russian culture exemplifies some of these practices.

Effective cultural training should focus on the development of cultural differences as a resultant of historical and social context. It is also critical to make it clear that differences that might be attributed to national culture may have roots in other attributes of the individual such as gender, profession, and social class.

James Kass

Dr. James Kass spoke as the representative of the European Space Agency (ESA). He described ESA as an inherently multi-cultural organization, noteworthy because it does not have a dominant national culture. Dr. Kass described simulation research that included a critical contextual element – the interface between crew and ground-based control. It was pointed out that the Russian space program places more emphasis on psychology than others – before, during and after flight. This includes psychological selection of crews for missions. Nevertheless, interviews confirm that there still is much incompatibility among crew members, and that these incompatibilities are associated with increased error and inefficiencies in operation.

This points to the necessity for further investigations into prophylactic psychological efforts such as team training. Providing the crew with the necessary tools to deal with interpersonal problems becomes of paramount importance especially for long-duration and distant flights (such as manned exploration of Mars) where ground intervention becomes less practical. However, unnecessary replication between agencies should be avoided in favor of collaboration. Investigations should be multi-organizational and multi-cultural in both planning and execution to achieve optimal results.

Laura Galarza

Dr. Laura Galarza presented research needs from the perspective of NASA's Operational Psychology program. Issues identified were congruent with those raised in other presentations. They included developing consistent definitions of constructs across cultures, optimizing leadership of international crews, organizational and national differences in mission management, gender issues, and the nature of interactions among national, organizational, and team cultures. Dr. Galarza also discussed the current initiatives within the Operational Psychology program in the area of cross-cultural training for prospective crews of upcoming ISS missions.

Further areas of investigation should explore the best methodologies for skill building and training, and determining which aspects of training should be culture-specific versus those that should be integrated. Differences in concepts of self-management and self-care should also be explored.

Christopher Flynn

Dr Christopher Flynn of the Johnson Space Center provided the psychiatric perspective based on his operational experience dealing with multi-national missions and the various national space agencies. He pointed out national and professional differences in diagnosis and treatment of medical and psychiatric problems, and discussed the current efforts of ISS partners to achieve a consensus in these areas.

He also described experiential training that exposes groups of astronauts/cosmonauts to challenging environments as part of mission preparations.

Robert Castle

Robert Castle, NASA Flight Director, briefed the workshop on the organization and management of multi-national International Space Station (ISS) missions. Much experience about multi-cultural operations has been gained through collaboration with Russia on MIR flights.

In contrast with NASA, Russian operations are more centralized, information tends to reside in people's heads rather than formal documentation, and individual relationships are the glue that binds the operation. The whole process takes more time. This observation reflected Bond's earlier comments, i.e., that cross-cultural encounters will initially appear less efficient because they are more time-consuming. It also reflected a basic difference between American and Russian perspectives on knowledge. As Dr Gushin commented, "In Russia, we say if it is important, it can not be written down".

Robert Castle also discussed specific differences in the command hierarchy between Mission control in Houston and the TSUP (Mission Control) in Moscow. In the ISS, a 'Lead' Mission Control Center (MCC) will perform all real-time operations and will be charged with directing the ISS crew. Each MCC will be run by a single Flight Director who will work directly with other Flight Directors. Members of the ISS team will work with their disciplinary counterparts, for example, environmental experts in Houston with their counterparts in Russian/Japanese/European MCCs. The complexity of coordinating

decentralized, national centers is recognized as a significant challenge for operations – with potential safety implications.

Astronauts – Ellen Baker, Shannon Lucid, Chiaki Mukai, and Michel Tognini

Four astronauts who have experience with multi-national space flight formed a panel and shared their experiences with the workshop. The challenges and rewards of multi-national operations were recognized. Culture and language were acknowledged as significant influences on mission enactment. In particular, long training programs in countries other than one's own, and the requirement to function socially and operationally for extended periods in a second language were discussed in detail. It was pointed out that being an astronaut and part of the culture of space is a life-changing event.

A program self-generated by the NASA astronauts and behavioral scientists to provide experiential training in teamwork and leadership was described. In this approach, astronauts participate in a two-day workshop introducing the themes of leadership, teamwork, and self-management using space experience and historical examples. Groups of six astronauts then attend field exercises in Northern Canada. This effort demonstrates recognition by the astronauts themselves of the importance of establishing a compatible mission culture to optimize performance and adjustment during long-duration missions.

Workshop Recommendations

The following are recommendations arising from two days of discussions by the workshop participants. Due to the broad nature of the topic, the brevity of the deliberations, and the diversity of the participants' cultural backgrounds (national, professional, and organizational), the attendees agreed that more than one brief workshop would be needed to develop a comprehensive agenda for research. To that end, the recommendations are offered as a starting point for further refinement.

Workshop participants agreed that the operational importance of cultural issues warrants their inclusion in the behavioral research agenda. While there is little hard data on the interplay of cultural factors in space missions and their management, a good deal is known about culture and behavior in other settings. The recommended program of cross-cultural investigations should fit in the category of action research, a systematic effort to collect valid data while using best available information to design and apply interventions to improve the operational environment rather than waiting for definitive answers from empirical research. For example, national differences in perceptions of appropriate leadership (consultative versus directive) and styles of communication (indirect versus direct) are known and sensitivity to such differences can be fostered in training using both didactic and experiential techniques. Similarly, although research into organizational cultures associated with mission management is urgently needed, ongoing efforts to harmonize diverse management approaches should have high priority. In the collaborative international atmosphere of current and future space endeavors, the panel stressed the need to avoid the unilateral imposition of one set of practices on all participating organizations (the dominant culture model).

The mandate of this workshop was specific in asking the panel to consider the role of culture in the long term research needs of space exploration, going beyond the International Space Station (ISS) currently under construction. However, the panel felt it important to stress the current role of ISS as both a research platform and a proving ground for culture-oriented research and countermeasures that can be derived from such research. In addition, the group identified known and potential problems related to culture and formed recommendations that appear below. These recommendations can be broadly divided into general, methodological, and specific recommendations detailing individual, crew, organizational, and psychiatric and medical factors warranting investigation.

I. General Recommendations

A. Include culture as a fundamental aspect of psychosocial research

Culture should be included as an element of an ongoing and coordinated research effort into other more broadly based issues. The separation of culture, *per se*, into its own research agenda is unnatural and likely to produce dubious findings. Current and future studies of behavior and performance, sociology, command and control and medical

support, for example, would benefit greatly from the inclusion of culture and culturally related factors as part of their context.

B. Stress multinational, collaborative research and the avoidance of ethnocentrism

Research designed and conducted within a single culture risks being ethnocentric in nature and drawing conclusions that will not generalize to the range of participants in future space missions. Investigations that address culture need, by definition, to be multinational and collaborative in nature. Currently, the majority of research is conducted mono-culturally and is thus plagued with conceptual problems. Fundamentally different theoretical foundations, differences in methodology, homogeneity of subject pools, and organizational specifics severely limit the utility and acceptance of countermeasures that stem from such research. An international research agenda paired with a collaborative research approach would go a long way towards minimizing such justified criticisms and producing more reliable recommendations for performance and safety enhancement

II. Methodological Issues

A. Use appropriate research subjects and venues

The appropriateness of the subjects used for research in support of long duration space flight will determine the utility of findings. Investigations of cultural issues relevant to space flight need to be conducted in settings and with subjects that will generalize to professionals working in stressful, isolated environments for extended periods of time. This requirement may change the nature of psychological research currently being conducted. Typically, undergraduate students serve as research subjects for the majority of current psychological and cultural studies. Far more relevant data will come from using subjects who represent professionals from the nations and disciplines collaborating in the International Space Station and future explorations. Of course, the most appropriate research population consists of astronauts, cosmonauts, and ground personnel involved in missions. Practically, however, it will be difficult to use this population to investigate all relevant questions and many investigations will need to employ analog groups and environments. Announcements of research opportunities should stress the criticality of subject selection and the importance of choosing appropriate of analog environments.

B. Address known complications of cross cultural research

Data collection methods will include surveys, interviews, ethnographic studies, systematic observations of individual and team behaviors, diaries, and a variety of unobtrusive behavioral indicators.

Among methodological challenges to cross-cultural research are issues of linguistic equivalence among people with differing primary languages. Another methodological problem comes from the existence of response biases that threaten the validity of conclusions drawn from comparison of the responses of members of different cultures to

questionnaires and interviews (Smith & Bond, 1999; Helmreich & Merritt, 1998). Members of some cultures eschew extreme responses to Likert-type probes in favor of more neutral responses, while those from other nations tend to favor more extreme endorsements. While not posing insurmountable problems, these are issues that need to be recognized and addressed by researchers dealing with multi-national respondents.

C. Address conceptual limitations of simulations

Simulations of space missions can provide important insights into crew dynamics and cultural issues, including how mission cultures develop. Simulations are most useful when participants are from the target populations or have similar professional and demographic backgrounds.

Simulations, however, pose their own methodological problems for psychological and cultural research. One of the greatest challenges is determining what tasks to have crews perform during simulations. Among the motivations for space flight are the opportunity to master new situations and to do meaningful work in a unique environment. Many activities can only be accomplished in the zero gravity environment of space. In contrast, many simulations give crews no meaningful work to do. The reactions of those whose only function is to be a psychological subject are unlikely to be isomorphic with those of individuals and teams doing important and self-rewarding tasks in a unique environment. Judgments will have to be made as to the appropriate length for a simulation to capture the dynamics of long duration space flight. It is highly unlikely that volunteers, even from the astronaut/cosmonaut corps, would be willing to endure an earth-based simulation of the same duration as a flight to Mars. In sum, simulation can provide data on cultural as well as other psychological aspects of individual and team function, but with serious limitations.

D. Respect the sensitivity and confidentiality of data

Astronauts, cosmonauts, and mission personnel can provide some of the most critical data on the interaction of cultural factors with other determinants of behavior and adjustment. Data provided by mission participants can yield insights into such critical issues as crew selection, mission composition, crew autonomy needs, and authority structures. Similarly, training exercises such as the wilderness experience developed by the astronauts can also provide valuable information about team dynamics and interpersonal issues in the most relevant population. On a cautionary note, experience in analog domains has emphasized the need to prevent the use of these data to jeopardize the careers of subjects participating in such research. When this occurs, valid data collection becomes compromised and research questions remain unanswered.

Mission personnel and managers from countries participating in space programs are likely to be concerned about cross-cultural comparisons. There is an unfortunate tendency for members of a particular culture to see their own values as 'correct.' Given NASA's central role in managing programs such as the ISS, those from other countries may fear that data will be used to denigrate their cultures and contributions. NASA sponsors and researchers must be sensitive to these justifiable anxieties, and ensure that such research is carried out in an unbiased and culturally sensitive manner.

III Specific Research Areas

Consideration of culture as context should be an explicit strategy for research into behavioral issues related to the human exploration of space. Cultural effects need to be included in research addressing individual, crew, and organizational issues in preparation for long duration, multi-cultural missions.

A. Individual level

1. The greatest challenge at the individual level is the selection of crew members who have psychological attributes enabling adaptation to lengthy, stressful missions involving isolation from earthly support. The ability to function effectively in a multi-cultural setting is but one of the many elements that need to be considered. Research should identify core personal values associated with adaptation to such settings.

2. Methods of training that would prepare individuals for functioning in multi-cultural endeavors (prior to crew assignment) need to be evaluated.

3. Beyond the initial stage of astronaut selection, research is required to further identify effective strategies for the ongoing psychological support of individual crewmembers. Investigations need to recognize cultural differences in preferred means of support and definitions of appropriate intervention. Analysis of past space flight experience, insight from analog studies, and an ongoing program of in-flight research are all potential lines of fruitful investigation in this area.

B. Crew level

1. At the level of a mission crew, a new challenge exists in the form of composing, from an existing pool of candidates with the requisite technical expertise, a multinational team who can work effectively as a group for an extended period. Cultural background and values should be investigated as part of this mix. Factors influencing the success of crew composition also include gender and individual and national differences in attitudes regarding appropriate roles and behaviors for the sexes.

2. Crew leadership is a critical aspect of mission design. As noted, large national differences exist in perceptions of the appropriate style of leadership and this should be investigated as an area to be harmonized across disparate team membership. Similarly, differences exist between cultures in perceptions of appropriate behavior for a subordinate team member.

3. Communications patterns are also critical to performance. In addition to barriers imposed by language, individuals from different national cultures may differ in the

style of speech they characteristically employ – for example, direct versus indirect styles that may lead to misunderstanding or perceptions of inappropriateness.

4. Perceptions of the importance of formal Standard Operating Procedures (SOPs) and rules and the need to follow them should be examined. This is an area where large national differences have been found in other domains. Anecdotal evidence suggests that there may be substantial differences in attitudes about the necessity for formal procedures among collaborating space programs. This is an area with high potential for conflict and misunderstanding.

5. Training strategies also merit investigation. How best should a multi-cultural crew be prepared psychologically for its mission? What kinds of experiential and didactic instruction are most effective and best received by participants from different cultures? Is formal training in the cultural values of participating crew members valuable or divisive?

6. It is important to investigate how crews develop their own "mission culture" from the diverse individuals selected. Can guidance be given to avoid conflict and the emergence of a "pathological" culture?

7. Studies are also needed of changes in group dynamics over time. Are there interventions that can enhance crew functioning? What sorts of work-rest schedules should be planned and how much flexibility should be planned?

C. Organizational level

Organizational factors are critical to mission success and are also the most sensitive to study since they touch on issues of national sovereignty and pride. Because there are not close analogs of the decentralized management of multi-national missions involving multiple national control agencies, thorough analyses of the successes and problems in the operational evolution of the International Space Station is essential. Such investigations would best be conducted by a multi-national research team independent of operational mission management. The following issues are of critical import and warrant investigation both in space operations and in analog environments.

1. What level of autonomy should be given to mission crews? This will be a significant organizational decision with implications for mission success and has been raised as a major concern by the Committee on Space Biology and Medicine of the National Academies of Science.

2. Managerial decision making processes also are in need of investigation. How does decision making and its associated communication function in a decentralized, multi-cultural endeavor.

3. Resolution of conflicts between ground and spacecraft and between the various ground agencies involved will also be a critical issue in future missions. Differences associated with national culture may play a substantial role in these processes.

4. Attitudes and practices of individual space agencies with regards to safety and risk should also be studied systematically. Such attitudes are known to vary widely between nations and have serious implications for the conduct of missions in this dangerous and technically challenging environment. This line of investigation should also include considerations of cultural differences in ergonomic standards and practices (e.g., differences in color-coding and shape and size of controls, etc.) that may affect safety.

D. Psychiatry and Medical operations

1. Although the distinction between medical/psychiatric support and the routine support of crews is artificial, given the extreme nature of space operations, the panel identified a lack of uniformity in diagnosis and treatment as an issue warranting special attention. This lack of uniformity is particularly problematic in the field of psychiatry. Recent efforts by working groups within the multinational ISS community were recognized by the panel as instrumental in assuring culturally sensitive and mutually agreeable protocols for the medical and psychiatric support of astronauts and cosmonauts on upcoming space flights. The panel strongly recommends that such initiatives are maintained and supported on an ongoing basis by all International Partners.

Conclusions

There was a strong consensus among the group, including the astronauts present, that cultural issues are highly relevant to safety and behavior on long duration multi-national missions. There was also agreement that research is needed to clarify relationships between and among national, organizational, and professional cultural values and the performance and psychological health of crews. However, it was also agreed that cultural research is not an end in itself for NASA sponsorship, but instead a contextual factor that needs to be included in the research agenda supporting the human exploration of space.

Including cultural issues as input factors influencing both team processes and mission outcomes has a number of implications regarding research methods, research settings, and the confidentiality of data. Cultural issues are of utmost sensitivity because of their threat to national, organizational, and individual self-concepts. It is essential that research and conclusions drawn from data collected respect the cultures of individuals and participating states. There was a consensus among the workshop attendees that future research in this area should be international and collaborative in nature.

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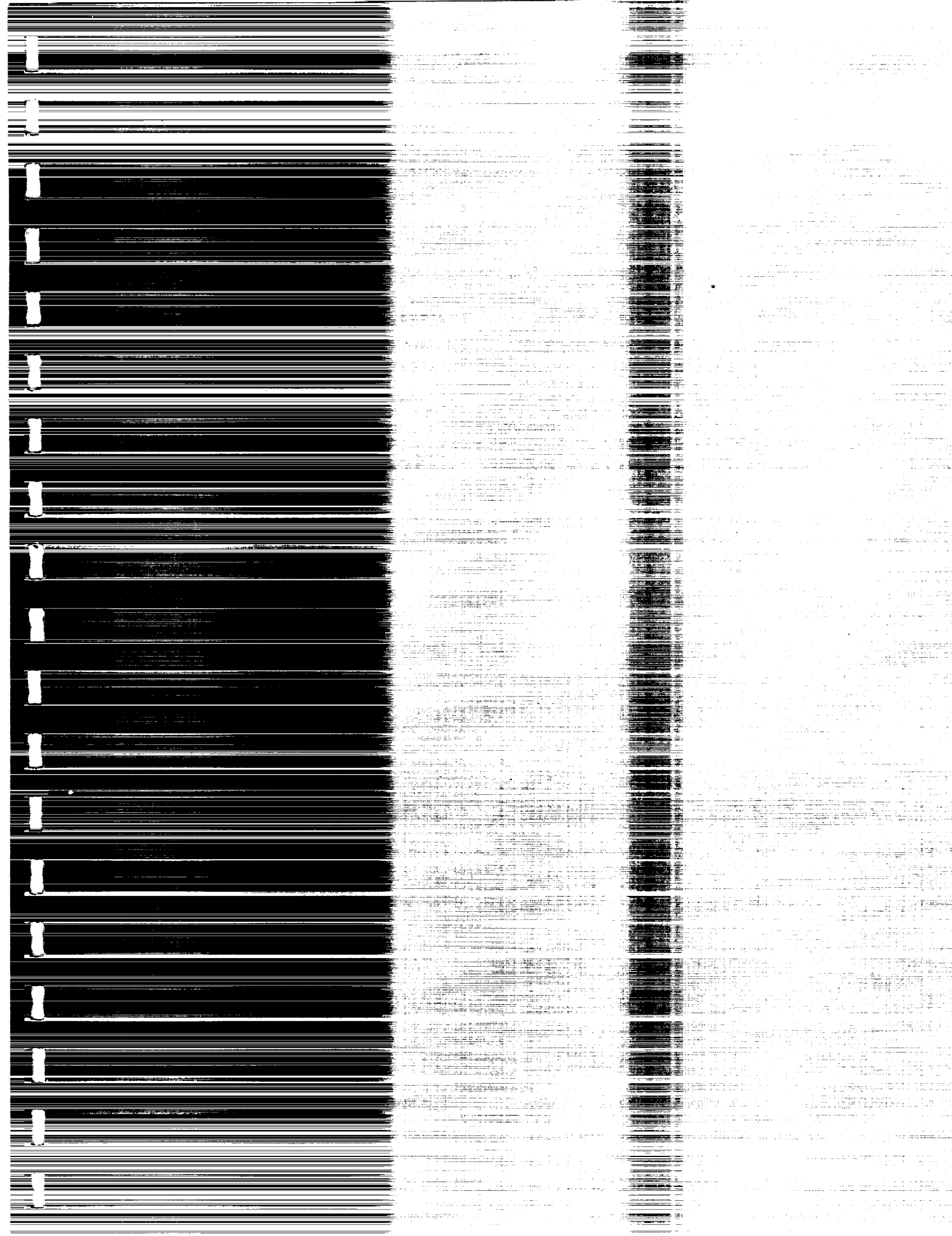
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NASA Johnson Space Center
&
National Space Biomedical Research Institute

SPACE HUMAN FACTORS & HABITABILITY WORKSHOP

April 18-19, 2000
Nassau Bay Hilton
Houston, Texas

AGENDA

Tuesday, April 18

8:00 a.m.	Welcome & Introductions	NASA/NSBRI
8:30	Workshop Purpose and Expected Products	Dane Russo
	Overview of Bioastronautics and NASA Space Human Factors & Habitability	
9:00	Research Functions	Tom Rathjen
9:15	Operations Functions	Dane Russo
	Graphics Research & Analysis Facility	Jim Maida
	Lighting Environment & Test Facility	Jim Maida
	Anthropometrics & Biomechanics Facility	Jim Maida
10:00	*BREAK*	
10:15	Usability Testing & Analysis Facility	Barbara Woolford
	Food Lab & Food Systems	Vickie Kloeris
	Operational Habitability	Jennifer Novak
	Habitability Design	Jan Connolly
	Human Engineering Integration	Becky Burnett
	Man Systems Integration Standards	Clete Booher
	Technology Transfer	Tico Foley
12:15 p.m.	*LUNCH*	

1:30	Overview of NSBRI	NSBRI
2:00	Consortium Institution Presentations (15 min. each)	
	Brookhaven National Laboratory	Drs. Gatley & Telang
	Harvard Medical School	Dr. Dennerlein
	The Johns Hopkins University	Drs. Hamill & Murphy
	Massachusetts Institute of Technology	Drs. Horgen & Newman
	Morehouse School of Medicine	Drs. Sonnenfeld & Tosini
	Baylor College of Medicine	Dr. Alford
3:30	*BREAK*	
3:45	Consortium Institution Presentations (continued)	
	Mount Sinai School of Medicine	Drs. Rifkin & Swartz
	Rice University	Dr. Byrne
	Texas A&M University	Drs. Clayton, Moore, Parke & Schuller
	University of Arkansas for Medical Sciences	Dr. Winter
	University of Pennsylvania Health System	Drs. Rogers & Szuba
	University of Washington	Dr. Schwartz
5:15	*ADJOURN*	

Wednesday, April 19

8:00 a.m.	Opening Remarks & Plan for the Day	NASA/NSBRI
8:15	General Discussion & Questions on Presentations	
9:15	Identify Areas of Cooperation: • Topics for which NSBRI is currently willing/able to provide cooperation, expertise • Specialties in which specific facilities & capabilities exist at NSBRI institutes	
10:15	*BREAK*	
10:30	Identify Specific Topics for Follow-up Activities & Appropriate Representatives for Various Areas of Cooperation	
11:15	Follow-Up Activities • Who - Responsible Persons – NASA/NSBRI • When – Schedule for Activities	
12:00	Closing Remarks	NASA/NSBRI
12:15 p.m.	*ADJOURN - LUNCH*	

National Space Biomedical Research Institute
SPACE HUMAN FACTORS AND HABITABILITY WORKSHOP
April 18-19, 2000
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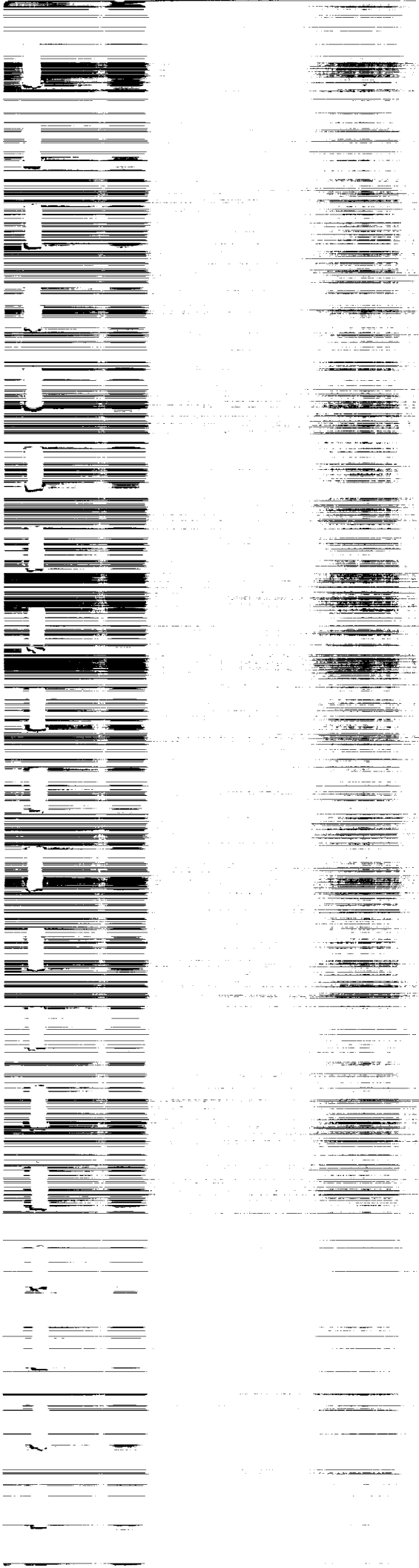
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Artificial Gravity Research Aboard the R2 Mission

Report From the Planning Meeting: April 4-5, 2000

Center for Advanced Space Studies, Houston, TX

Background:

NSBRI has been requested by NASA to propose a pilot study, or pilot studies, that will supplement the initial NASA space flight test of the human centrifuge as a shuttle Detailed Test Objective (DTO). The objective of the NSBRI pilot study is to better understand the physiological effects of intermittent exposure to artificial g combined with exercise in microgravity. This effort may lead to further study of intermittent centrifugation as a countermeasure. In addition to the pilot study effort, the NSBRI should propose and outline a long-term effort in the area of artificial g. This proposal should address an integrated and comprehensive approach to the issues associated with dose response to various levels of g from microgravity to 1 g. The NSBRI should consider continuous versus intermittent application of artificial g, both passive and with various forms of exercise. A long-range plan for countermeasure development in the area of artificial g should be developed in draft form for NASA review by May 15, 2000.

Goals:

1. Develop plan for artificial gravity pilot study aboard the R2 mission.
2. Begin to develop long-range plan for comprehensive study of artificial gravity as a multi-system countermeasure.

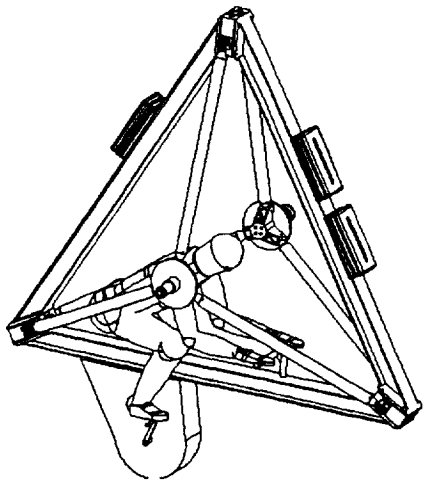
AG Pilot Study for R2 (and Subsequent) Missions

Flight Experiment Goals

- Develop and flight test a human short arm centrifuge facility versatile enough to allow flight investigations of the critical questions that must be answered to develop a prescription for AG as a multi-system countermeasure.
- Demonstrate the feasibility of using centripetal acceleration as gravity replacement therapy during space flight by studying the operational impacts and physiological effects of repeatedly exposing crewmembers to an AG/exercise paradigm during flight.
- Test the hypothesis that AG plus moderate aerobic exercise will maintain aerobic capacity in crewmembers aboard a 16 day space flight mission.

Hardware Requirements

Several short-arm centrifuge design concepts are available for potential flight on the R2 mission. The design proposed by the JSC Engineering group (see figure) is representative.



The following requirements should be designed into whichever design is selected for the R2 mission:

location: the optimal location for the centrifuge appears to be having it mounted on the aft bulkhead of the SpaceHab module (two racks and all aft stowage containers would have to be removed to accommodate the centrifuge)

radius: the centrifuge radius should be the maximum achievable within the SpaceHab aft bulkhead area (96–108 inches)

primary subject orientation: maximizes cardiovascular and musculo-skeletal effects by recreating (within geometric limitations) upright terrestrial gravitational loading (i.e., centrifugal force vector aligned with $-g_z$ body axis); probably best achieved with head on or near the center of rotation, seat and feet near the perimeter of the rotational envelope, centrifuge rotation axis parallel to the subject's interaural axis (pitch plane rotation)

secondary subject orientation: focuses on neuro-vestibular system by providing tonic otolith organ stimulation; possibly achieved with a 45 degree recumbent position, similar to that conventionally used for recumbent cycling; this would move the head off axis by roughly the distance from the seat to the head–about 3 feet; it would also accommodate larger subjects

built-in exercise device: in the interest of safety (minimizing the risk of per-rotatory syncope) and optimizing crew time utilization the facility should provide for lower body exercise during rotation; this could be achieved using a centrifuge-mounted cycle ergometer or equivalent, possibly with a special cam or vibrating foot support system to promote bone mineral retention. Both the primary (head on center) and secondary (head off center) subject orientations could be achieved with the seat and pedals in one place, simply by providing the capability to reposition the rider's torso back by 45 degrees from the axis of rotation.

subject restraint system: a comfortable, quick-release restraint system must be provided to protect the subject from injury during rotation:

1. the subject's torso must be secured to the seat/backrest , perhaps using a three- or five-point harness
2. the subject's feet must be secured to the pedals of the exercise system during exercise, perhaps using toe clips
3. motions of the subject's arms, legs, hands, and feet must be limited, perhaps using tethers, to remain within the safe operating envelope of the centrifuge
4. two modes of restraint are required for the subject's head: a static mode that completely immobilizes the head and a dynamic mode that allows the subject to make free head movements in the plane of centrifuge rotation. The dynamic mode might be achieved using a head goniometer system similar to that made by Puppetworks (www.puppetworks.com), which allows tracking of pitch head movement is surprisingly comfortable and nonencumbring. The static mode could be achieved by a variation on the basic Puppetworks design that would allow locking the goniometer joints when head movements are not desired.
5. A shroud may be required to eliminate nauseogenic, optokinetic visual stimuli; if included, a fan may be required to reduce heat load

angular velocity: sufficient to provide up to 3 g centrifugal force at the perimeter

angular acceleration: not critical; nominal ramp-up and ramp-down accelerations of 10–30 deg/sec^2 would be sufficient; an emergency braking system must be available to stop the centrifuge within 15-20 seconds

experiment duration: expected to be 60 minutes per subject; constant velocity must be maintained over this period

monitoring (safety): blood pressure, heart rate (ECG), surveillance video, bi-directional audio communication, cardiovascular life support kit (defibrillator, etc.)

monitoring (science): eye movements, head movements, load, torque, and/or work monitor on ergometer device, oxygen consumption (desired, not required)

In-Flight Protocol

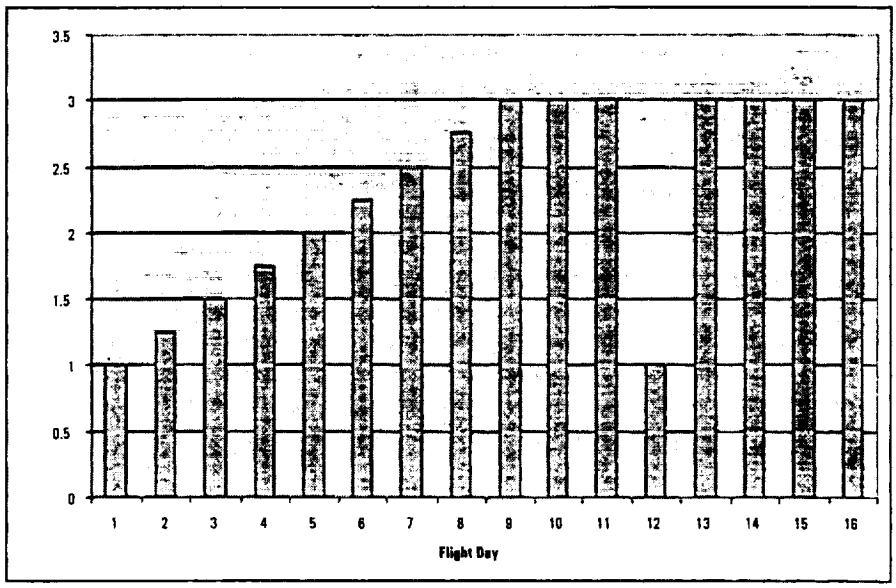
test subjects:

1. It is assumed that six (or seven) crewmembers will be assigned to the mission and that all will participate as subjects.
2. Four of the crewmembers will be asked to serve as experiment subjects, and the remaining two (or three) will be asked to serve as control subjects

experiment subject protocol:

1. Each experiment subject will spin in the centrifuge for one hour during each day of the mission beginning on Flight Day 1 (launch is on Flight Day 0).

2. Centrifugal force loading will begin on Flight Day 1 with 1.0 g at the rim of the centrifuge, and will increase by 0.25 g/day until Flight Day 9, when the loading at the rim will be 3.0 g (see figure). The 3.0 g load will be used from Flight Day 9 until the end of the mission, except on Flight Day 12, when a 1.0 g load will be used.



3. Experiment subjects will be required to perform continuous, moderate exercise throughout each one-hour spin, except on Flight Day 12. Exercise will be performed on the centrifuge-mounted treadmill according to a pre-specified workload schedule.
4. Flight Day 12 will be reserved for a test of orthostatic intolerance: each experiment subject will spin for one hour under a 1.0 g load (at the rim) without exercise.
5. A test operator will be required to assist and monitor the test subject throughout all centrifuge spins.

control subject protocol:

Each control subject will perform one hour of continuous, moderate exercise during each day of the mission, except on Flight Day 12. Exercise will be performed on the centrifuge-mounted treadmill according to a pre-specified workload schedule. The centrifuge will remain stationary during these exercise periods.

in-flight monitoring:

1. In-flight monitoring will primarily be focused on subject health and safety.
   - Blood pressure and heart rate (ECG) will be monitored continuously throughout centrifugation. While it may be necessary to telemeter these data to an operator workstation, it is expected that standard alarms will suffice.
   - Surveillance video and bi-directional electronic audio communication will be required if the subject must be placed within a shroud or is otherwise obscured from the sight of the operator.
   - A cardiovascular life support kit (defibrillator, etc.) will be flown for emergency.
2. In-flight science monitoring will include recordings of:
   - Blood pressure and heart rate
   - Work output of the ergometer device
   - Angular velocity and/or g-level of the centrifuge
   - Eye movements, head movements, and oxygen consumption are desired, but not required

In-flight Supporting Studies

No in-flight supporting studies are proposed by NSBRI; however, several on-going NASA studies might provide useful adjunct data relevant to the efficacy of the specific AG protocol as a countermeasure. These include:

- Studies of Ca^{++} balance as an indicator of bone loss
- Studies of protein turnover as an indicator of muscle wasting

- Studies of renal stone formation

Pre- and Postflight Studies

1. The primary focus of the scientific investigation will be to evaluate the protective (countermeasure) effects of AG on postflight health, safety, and performance of crewmembers. To that end, a wide range of pre- and postflight measurements will be proposed.
2. To ensure the widest possible participation, proposals for pre- and postflight supporting studies will be solicited from the general scientific community and selected through a rigorous peer-review process. Areas of interest include:
   - Cardiovascular: aerobic capacity, orthostatic tolerance
   - Muscle: mass, strength, composition
   - Bone metabolism: Ca^{++} balance, renal stone risk (note that it is unlikely that measurable bone mineral density changes (DEXA) will be observed after only 16 days in space)
   - Neuro-vestibular function: balance/gait control, head-eye coordination, gaze control, dynamic visual acuity
3. Investigators proposing techniques that already have sufficient space flight data to allow comparisons between the AG subjects and normal (non AG) astronaut populations will be given priority for selection.

Ground-Based Supporting Studies

1. A ground-based supporting study will be performed prior to flight.
2. Owing to insurmountable confounds (on subject deconditioning and centrifuge utilization) created by the terrestrial gravitational field, it will not be possible to demonstrate unequivocally in a ground-based paradigm that the proposed in-flight protocol will protect cardiovascular, muscle, bone, or neurovestibular function. Thus, the focus of the ground-based study will be primarily on safety issues related to spinning partially deconditioned subjects in the short-arm centrifuge.
3. The ground-based paradigm will use bed-rest as a model of space flight deconditioning
4. Three groups of subjects will be studied during 16 days of bed-rest:
   - Group 1: sedentary–this control group will be limited to strict bed-rest for 16 days without exercise or centrifugation
   - Group 2: exercise–this control group will remain in bed for 16 days, but will perform one hour/day of moderate supine ergometer exercise according to the same workload schedule that control crewmembers will follow
   - Group 3: centrifuge–this experiment group will remain in bed for 16 days, but will be transported to a horizontal centrifuge/ergometer device to perform one hour/day of moderate supine ergometer exercise coupled with z-axis centrifugal force according to the same centrifuge loading and ergometer workload schedule that experiment crewmembers will follow
5. All investigations selected for pre- and post space flight studies will be performed on the bed-rest subjects as well.

Follow-on Studies

1. One flight will not be sufficient to test the centrifuge countermeasure.
2. At least two follow-on flights, similar in scope, will be required to:
   - Examine the effects of vestibular stimulation: repeat the same paradigm with the head off-axis and/or with head movements in the plane of rotation.
   - Examine the effects of centrifugation on postflight orthostatic intolerance: repeat the same paradigm without exercise.
   - Increase the n and/or fine tune the prescription
3. The order/priority/necessity of examining these issues will be determined after reviewing the results from the R2 mission.

Long-Range Plan for AG Research

Overview

1. Rotational AG has the potential to serve as a multi-system countermeasure, mitigating bone loss, muscle atrophy, cardiovascular deconditioning, and sensorimotor/neurovestibular adaptations.
2. A rigorous, peer-reviewed research program will be developed to answer the critical questions identified by the 1999 Artificial Gravity Workshop held in League City, TX (see below) and to investigate rotational AG as multi-system countermeasure during long-duration, exploration-class space flight. Fundamental ground- and flight-based research will first define operational parameters and then sustain development of an appropriate countermeasure.
3. Since there is much to learn about AG, and since AG may be most effective when combined with supplemental exercise or other countermeasures, the AG research project will not preclude research or development of other promising countermeasure concepts presently under consideration; the funding priority of AG countermeasure research may increase as potential side effects, design impacts, costs, and operational considerations have been sufficiently addressed.
4. While theoretical and animal models are valuable in any research process, they cannot substitute for systematic ground- and flight-based studies of the human response to AG; thus, the focus of this project will be on human studies.
5. Standards for operational performance during space flight will be established. Such metrics will not only direct studies of an AG countermeasure, but will also refine the analysis of existing countermeasure approaches.

Fundamental Goals

1. Implement a rigorous, coordinated, peer-reviewed research and development project to investigate rotational AG as a multi-system countermeasure against the detrimental health and performance effects of long-duration, exploration-class space flight.
2. Determine the optimal design characteristics for an AG countermeasure facility that will best promote human health and performance. Advocate multidisciplinary investigator teams.
3. Support the upgrade of existing ground and flight research sites and facilities as needed to perform fundamental research and development activities.
4. Promote the participation of and communication among all concerned, including experts from bone, muscle, cardiovascular, and neurovestibular fields, human factors, crew training, crew operations, and rehabilitation experts, and mission and vehicle designers.

Critical Questions

1. What relationships exist between operational performance and *continuously* applied AG (between 0 g and 1 g)? How does supplemental exercise affect these relationships?
   - What minimum continuous GIF would be required to maintain operational performance during interplanetary travel to Mars?
   - What type and level of AG, if any, would be required during an eighteen-month stay on the Martian surface?
2. What relationships exist between operational performance and *intermittently* applied AG?
   - What optimal duty cycle would be required to maintain operational performance during interplanetary travel to Mars?
   - How do different duty cycles affect these relationships?
3. What are the acceptable ranges of radius and angular velocity required to maintain operational performance in a rotating spacecraft? What are the optimal ranges for these same parameters?
4. What is the human capacity for dual adaptation, and how can the transition process between different GIF environments be investigated systematically?
5. Can the cardiovascular system dual-adapt to different GIF environments?
6. Can the central nervous system dual-adapt to different GIF environments? Can it adapt to/from the complex force environment within a rotating spacecraft?
   - What restrictions on orientation and/or movement within the rotating vehicle would simplify the adaptive processes of the central nervous system?

7. What are quantifiable standards for operational performance during a mission? What are the limits for degradation of the specific systems during various phases of a mission to Mars?

Implementation Plan

1. Establish a cross-disciplinary working/advisory group on Artificial Gravity. (completed 1/00–4/00)
2. Identify potential flight opportunities for deploying a short-arm human centrifuge aboard the Space Shuttle. (First potential opportunity identified as R2 mission in 11/01, with follow-on possibilities aboard Rn missions expected approximately every 12-18 months after R2.)
3. Design the in-flight protocols to be used during shuttle missions. (completed 4/00)
4. Establish peer-review process for shuttle studies.
5. Solicit, peer-review, and fund proposals for shuttle studies.
6. Identify and provide funding for upgrading, maintaining, and operating key ground-based AG facilities.
7. Solicit, peer-review, and fund "parametric" research and development activities aimed at defining AG prescriptions that will maintain the bone, muscle, cardiovascular, and neuromotor functions required during exploration-class missions. Through this process, ground-based research projects should:
   a. Establish fractional g amplitude requirements for continuous AG.
   b. Establish radius-rotation rate requirements for continuous AG, with a specific sensitivity to neuromotor function.
   c. Establish the optimal AG characteristics (duty cycle, amplitude, radius, and rotation rate) for intermittent AG. These characteristics should necessarily include prescriptions for promoting dual-adaptation to the AG and non-AG environments.
8. Develop hardware specifications and science plan for evaluation of AG aboard ISS–focus specifically on extended radius centrifuge solutions.
9. Provide AG recommendations/requirements to Mars mission and vehicle designers.
10. Solicit, develop, and perform ISS AG experiments with human-rated centrifuges.
11. Answer the critical questions necessary to make a go/no-go decision for a 2014 Mars mission, based upon ground-based and flight studies already initiated.

Secondary Goals

1. Evaluate the degree to which critical AG questions can be addressed using the ISS animal centrifuge. Modify and/or expand the planned program to include specific AG research objectives and then solicit relevant research proposals.
2. Establish a joint NASA/NIH research initiative to investigate the use of centrifuge devices in treating clinical populations (e.g., osteoporotic patients). Solicit research proposals against these objectives.

Appendix 1: R2 Workshop Agenda

April 4, 2000 Device Selection/Specification

0800–1200 Hardware Review ***Bill Paloski***

0800: SpaceHab Accommodation Constraints — Ken Jackson
0900: Human Powered Centrifuge Capabilities/Status — Bill Schneider & Chris Hansen
1000: Neurolab Centrifuge Capabilities/Status — Steven Moore
1100: Irvine Centrifuge Capabilities/Constraints — Arthur Kreitenberg

1200–1300 Lunch

1300–1700 Science Requirements ***Larry Young***

1300: Desirable Specifications
1400: Group Discussions
1600: Consensus Development

April 5, 2000 Protocol Development

0800–0900 Develop a Long-Term Plan ***Bill Paloski***

- Develop the big picture for flight research requirements
- Determine the requirements/role/timing for supporting ground-based research

0900–1200 Develop the Baseline Protocol ***Larry Young***

3. Define the AG Characteristics (g-level, subject orientation, exposure duration & frequency, etc.)
4. Monitoring Requirements/Peri-Rotation Dependent Measures

1200–1300 Lunch

1300–1500 Develop the Solicitation ***Ron White***

5. Rationale for R2 Mission
6. Call for Pre/Postflight Dependent Measures
7. Define review/selection processes
8. Define schedule

Appendix 2: R2 Workshop Attendees

1.	Bob Alford	NSBRI/BCM	
2.	John Charles	NASA/JSC	jcharles@ems.jsc.nasa.gov
3.	Bernard Cohen	NSBRI/Mt. Sinai	bernard.cohen@mssm.edu
4.	Marty Fettman	NSBRI/Colorado State	mjf@lamar.colostate.edu
5.	John Greenleaf	NASA/ARC	jgreenleaf@mail.arc.nasa.gov
6.	Michael Grande	SpaceHab, Inc.	mgrande@spacehab.com
7.	Chris Hanson	NASA/JSC	chanson@ems.jsc.nasa.gov
8.	Heiko Hecht	NSBRI/MIT.	hecht@space.mit.edu
9.	Ken Jackson	SpaceHab, Inc.	kjackson@spacehab.com
10.	Arthur Kreitenberg	UC Irvine	akreit@msn.com
11.	Adrian LeBlanc	NSBRI/BCM	aleblanc@bcm.tcm.edu
12.	Steven Moore	Mt. Sinai	steven.moore@mssm.edu
13.	Chuck Oman	NSBRI/MIT	cmo@space.mit.edu
14.	Bill Paloski	NASA/JSC	wpaloski@ems.jsc.nasa.gov
15.	Sam Pool	NASA/JSC	sam.l.pool@jsc.nasa.gov
16.	David Rary	Boeing Huntsville	David.Rary@HSV.Boeing.com
17.	Chuck Sawin	NASA/JSC	csawin@ems.jsc.nasa.gov
18.	Bill Schneider	NASA/JSC	wschneid@ems.jsc.nasa.gov
19.	Suzanne Schneider	NASA/JSC	sschneid@ems.jsc.nasa.gov
20.	Cameron Sumner	SpaceHab, Inc.	csumner@spacehab.com
21.	Leland Stone	NASA/ARC	lstone@mail.arc.nasa.gov
22.	Bill Thornton	UTMB	
23.	David Tomko	NASA/HQ	dtomko@hq.nasa.gov
24.	Ron White	NSBRI/BCM	rwhite@bcm.tmc.edu
25.	Dave Wolf	NASA/JSC	dwolf@ems.jsc.nasa.gov
26.	Scott Wood	NSBRI/BCM	sjwood@bcm.tmc.edu
27.	Larry Young	NSBRI/MIT	lry@mit.edu
28.	Janice Yelle	NASA/JSC	jyelle@ems.jsc.nasa.gov

Appendix 3:

Ad Hoc Artificial Gravity Discussion Group

Minutes: 1/11/00 Meeting

Del Lago Resort, Montgomery, TX

An ad hoc group of NSBRI and NASA scientists, chaired by NSBRI director Larry Young, met from 8:00–10:15 p.m. to discuss a possible near-term opportunity to perform an Artificial Gravity (AG) experiment aboard the shuttle.

Attendees:

Ken Baldwin	NSBRI/UC Irvine	kmbaldwin@uci.edu
Bernard Cohen	NSBRI/Mt. Sinai	bernard.cohen@mssm.edu
Richard Cohen	NSBRI/MIT	rjcohen@mit.edu
William Evans	NSBRI/Arkansas	evanswilliamj@exchange.uams.edu
Marty Fettman	NSBRI/Colorado State	mjf@lamar.colostate.edu
Charlie Lundquist	NASA/JSC	clundqui@jsc.nasa.gov
Dan Merfeld	NSBRI/MEEI/Harvard	danmerfeld@mee.harvard.edu
Chuck Oman	NSBRI/MIT	cmo@space.mit.edu
Bill Paloski	NASA/JSC	wpaloski@ems.jsc.nasa.gov
Sam Pool	NASA/JSC	sam.l.pool@jsc.nasa.gov
David Tomko	NASA/HQ	dtomko@hq.nasa.gov
Conrad Wall	NSBRI/MEEI/Harvard	cwall@mit.edu
Scott Wood	NSBRI/Baylor	sjwood@bcm.tmc.edu
Larry Young	NSBRI/MIT	lry@mit.edu

Background:

1. Dr. Young drew everyone's attention to the following recommendations from last year's AG workshop:

Implementation Objectives

Immediate (0–6 months)

3. *Identify potential flight opportunities for deploying a short-arm human centrifuge aboard the Space Shuttle, including the human-powered centrifuge or the Neurolab centrifuge. Concurrently, initiate a process to design, develop, and peer-review pilot studies. These flight studies will characterize the physiological effects of g transitions that are encountered during intermittent rotation.*
4. *Implement a peer-review process and/or peer-review guidelines for "parametric" research and development activities. Prescriptions for both intermittent and continuous AG should maintain the bone, muscle, cardiovascular, and neuromotor function required during exploration class missions. Call for ground-based research proposals[1] to:*

[1] Note that concerns were raised regarding our ability to extrapolate from animal models to humans. While animal experiments should be considered, caution must be exercised in this regard.

- *Establish fractional g amplitude requirements for continuous AG.*
- *Establish radius-rotation rate requirements for continuous AG, with a specific sensitivity to neuromotor function.*
- *Establish the optimal AG characteristics (duty cycle, amplitude, radius, and rotation rate) for intermittent AG. These prescriptions should necessarily include prescriptions for promoting dual-adaptation (to the AG and non-AG environments).*

2. Dr. Paloski informed the group that NASA/JSC/HSLSPO has begun planning for a possible "gap-filler" space shuttle science mission that, if real, would fly in about 2 years. HSLSPO management is recommending to HQ that the human life sciences focus for this mission be countermeasures, and, along with a number of previously selected countermeasure-oriented experiment proposals, have included on their preliminary recommendation list a human powered centrifuge.
3. Dr. Pool described the origin of the human powered centrifuge concept and the current status of a flight unit development effort.
4. Dr. Cohen (Bernard) described the STS-90 (Neurolab) human centrifuge, as well as some interesting findings from his experiments using that centrifuge.
5. Dr. Young then challenged the group to discuss/debate this potential opportunity. He led the group through discussions of 1) the risks and benefits of flying an AG facility/experiment in space in the near-term, 2) the characteristics that a near-term, shuttle-based AG facility should have to enable concept demonstration and/or feasibility testing of intermittent AG as a multi-system countermeasure, and 3) the process that should be used to determine how to use the facility will be used scientifically.

Recommendations:

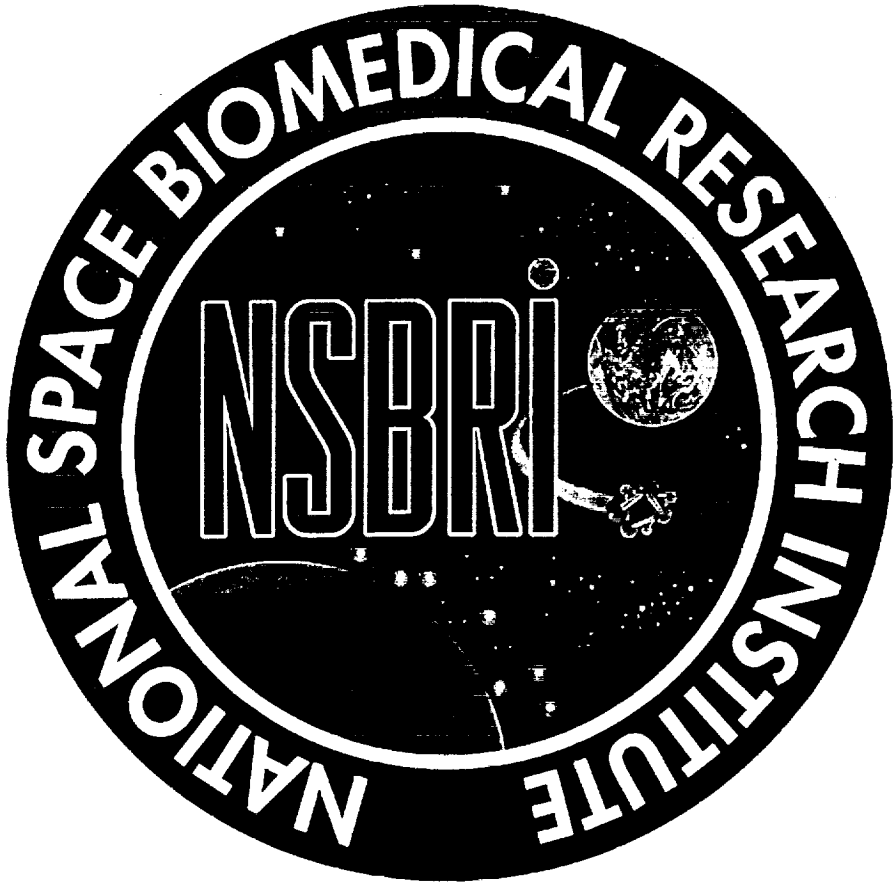
6. The group supports the notion of flying an early, shuttle-based AG facility for the purposes of demonstrating the concept and testing the feasibility of intermittent AG as a multi-system countermeasure. The target systems for short-duration crew will include:
   - cardiovascular: eliminating postflight orthostatic intolerance
   - sensory-motor/neurovestibular: eliminating postflight disturbances in balance control and oculomotor function, as well as Earth-readaptation sickness
   - musculo-skeletal: eliminating loss of mass and strength in the antigravity muscles and loss of bone mineral
7. The group recommends that the AG facility include the following capabilities:
   - primary subject orientation: maximizes cardiovascular and musculo-skeletal effects by recreating (within geometric limitations) upright terrestrial gravitational loading (i.e., centrifugal force vector aligned with $-g_z$ body axis); probably best achieved with head on or near the center of rotation, feet at the perimeter of the rotational envelope, rotation about the interaural axis (pitch plane)
   - secondary subject orientation: focuses on neuro-vestibular system by providing tonic otolith organ stimulation; possibly achieved with feet at or near the center of rotation, head at the perimeter of the rotational envelope, rotation parallel to the interaural axis (pitch plane) or with a variant of the primary orientation with the head moved some radial distance away from the center of rotation
   - alternate subject orientation: focuses on reproducing findings from the STS-90 centrifuge experiments; achieved by placing the subject at the perimeter of the rotational envelope with

long body (z) axis oriented parallel to the axis of rotation and with face toward or away from the direction of rotation (tangential orientation)

- exercise: in the interest of safety (minimizing the risk of per-rotatory syncope) and optimizing crew time utilization the facility should allow lower body exercise during rotation; this could be achieved using a centrifuge-mounted cycle ergometer or equivalent, possible with a special cam or vibrating foot support system to promote bone mineral retention
- angular velocity: sufficient to provide up to 2 g centrifugal force at the perimeter
- angular acceleration: not critical; 10–30 deg/sec^2 would be sufficient
- experiment duration: expected to be circa 30 minutes; system should be capable of continuously rotating a test subject for up to 60 minutes
- monitoring (safety): blood pressure, heart rate (ECG), surveillance video, bi-directional audio communication.
- monitoring (science): eye movements, head movements, leg plethysmography

8. The group recommends that AG facility users be selected through an open solicitation for proposals sponsored by NSBRI. The solicitation should be announced as soon as practical, and proposal preparation time should be as short as possible (4-6 weeks). Proposals should be rigorously peer-reviewed for scientific merit, and should be selected based on their potential for demonstrating the concept and testing the feasibility of intermittent AG as a multi-system countermeasure within the available space flight resources.
9. The group recognized that AG is a cross-cutting countermeasure that has high importance to multiple disciplines. They recommend that NSBRI:
   - develop and maintain a multi-disciplinary AG working group
   - develop a ground-based AG research program

Appendix C
Final Program & Project Reports
for the
Initial NSBRI Research Program
October 1, 1999 – September 30, 2000

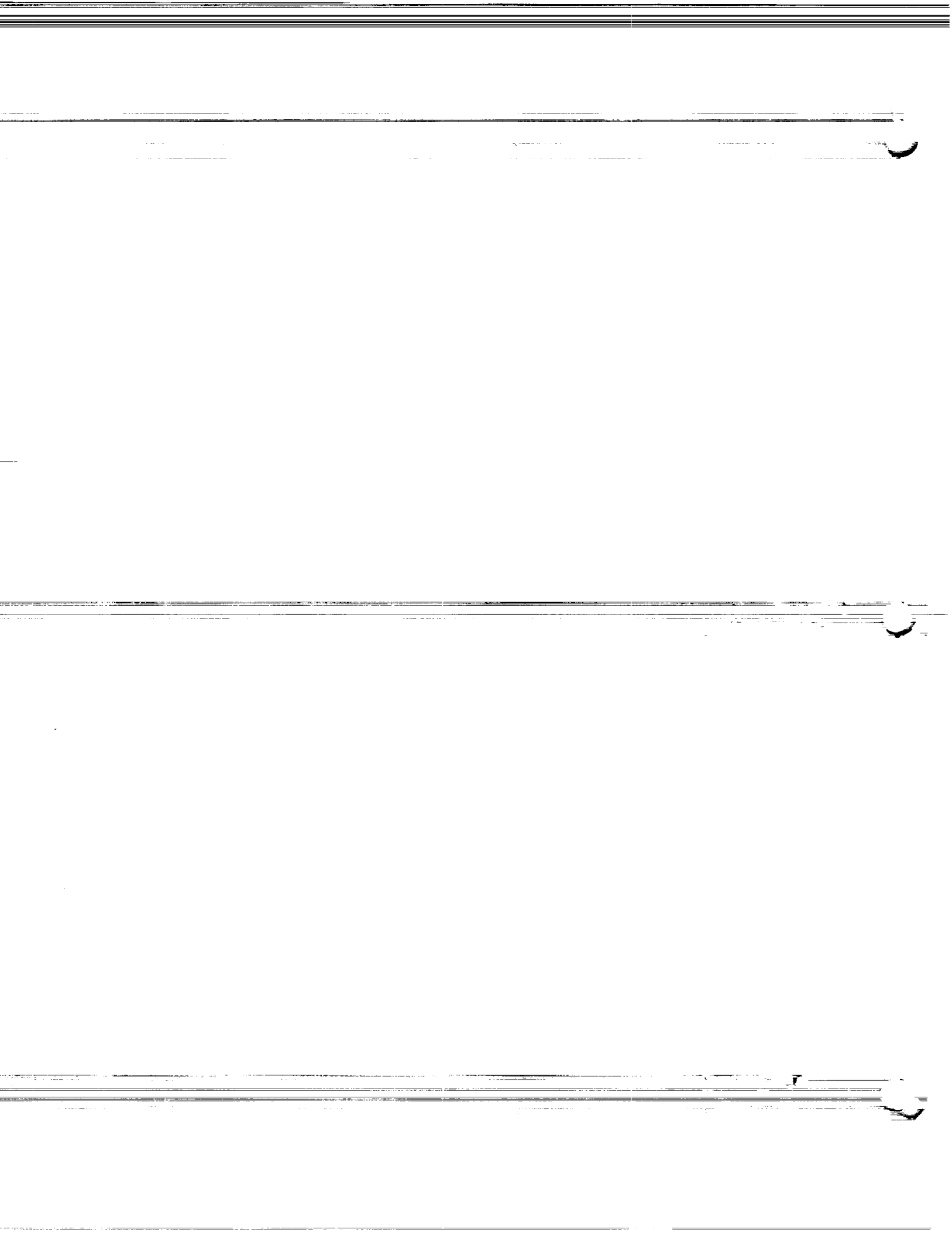


Volume One:
Bone Loss
Cardiovascular Alterations
Human Performance Factors
Immunology, Infection & Hematology
Muscle Alterations & Atrophy

NSBRI RESEARCH PROGRAM
BONE LOSS

Team Leader: **Shapiro, J. R.** — **Uniformed Services University of the Health Sciences (USUHS)/ Walter Reed Army Medical Center**

O'Malley, B. W.	**PI**	**Baylor**	**Novel Receptor-Based Countermeasures to Microgravity-Induced Bone Loss**
Brown, E. M.	CO-I	Harvard	
Bloomfield, S. A.	CO-I	Texas A&M	
Smith, C. L.	CO-I	Baylor	
Weigel, N. L.	CO-I	Baylor	
Bloomfield, S. A.	**PI**	**Texas A&M**	**Bone Blood Flow During Simulated Microgravity: Physiological and Molecular Mechanisms**
Delp, M. D.	CO-I	Texas A&M	
Hogan, H.A.	CO-I	Texas A&M	
Suva, L. J.	CO-I	SmithKline Beecham	
Turner, R. T.	CO-I	Mayo Clinic	
Schultheis, L. W.	**PI**	**Wash. Hosp.**	**The Effects of Partial Mechanical Loading and Ibandronate on Skeletal Tissues in the Adult Rat Hindquarter Suspension Model of Microgravity**
Bloomfield, S. A.	CO-I	Texas A&M	
Fedarko, N. S.	CO-I	Hopkins/SOM	
Ruff, C. B.	CO-I	Hopkins/SOM	
Shapiro, J. R.	CO-I	USUHS	
Thierry-Palmer, M.	CO-I	Morehouse	
Ruff, C. B.	**PI**	**Hopkins/SOM**	**Skeletal Structural Consequences of Reduced Gravity Environments**
Beck, T. J.	CO-I	Hopkins/SOM	
Newman, D. J.	CO-I	MIT	
Oden, Z. M.	CO-I	UT-Houston	
Schaffner, G.	CO-I	MIT	



NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

BONE LOSS TEAM

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EXECUTIVE SUMMARY

The National Space Biomedical Research Institute was created in 1997 with the intent of developing new and effective countermeasures to health risks anticipated during prolonged exposure to microgravity. Bone loss during extended spaceflight is a major risk to astronaut health and safety. The extent of bone loss has been documented based on bone density measurements obtained before and after several flights of varying duration. These include data obtained from the Gemini series, Skylab flights, Souyez flights and more recently from the Mir cosmonauts. In addition to bone density measurements, variations in serum and urine biomarkers of bone turnover and calcitropic hormones have been assayed. These results indicate that bone loss approximates 1-2% per month with sizable variation by individual and bone site measured, that both increased bone resorption and decreased bone formation contribute to the decrease in bone mass, and that it has not been possible to minimize bone loss with various exercise programs or dietary supplements.

The consortium of 7 universities which initially formed NSBRI was selected in 1997. Following this selection, several research protocols directed at countermeasure development against bone loss were competitively reviewed and four projects were selected to receive funding for the first three years of the program. This report reviews the accomplishments of the investigators involved in this funding cycle and will focus on how these contribute to the development of effective countermeasures against bone loss during extended spaceflight.

I. PROGRAM RESEARCH ACCOMPLISHMENTS

Program Synergy: Four research projects were initially approved for funding by NSBRI. Collectively the projects address the modulating effect 3 receptor agonists exert at the osteoblast and osteoclast level, specific mechanical factors (blood flow and mechanical loading) acting on bone cells to influence rates of bone resorption and the development of analytic methods, based on DEXA, to assess bone mass and thus fracture risk in the femur. The questions initially posed by these projects may be summarized as follows:

1. Considering the essential role of hormone receptors in cell differentiation and function, what novel pharmacological agents can be developed to diminish osteoclastic bone resorption and to increase osteoblastic bone formation during, and after exposure to microgravity ? .

2. To what extent does decreased blood flow increase bone loss in the lower extremities during weightlessness?

3. What level of mechanical loading is required to maintain normal bone

remodeling during microgravity conditions, and will pharmacologic intervention act synergistically with mechanical loading to limit bone loss?

4. How can net bone loss during microgravity be assessed in a timely manner, and what information does this provide about mechanisms facilitating bone loss and fracture risk?

Research Synergy

The projects reported here address the development of countermeasures from the cellular level (receptors) to the organ level (blood flow, vascular responses), to mechanical stimulation and antiresorptive therapy for the bone system, to the potential for real time assessment of changes in bone mineral density and bone structural changes. At each level, these investigations are interdependent as illustrated by the utility of measuring real time change in bone density applied to the effects of specific countermeasures tested during flight.

Research Accomplishments

Receptor Agonists: Three receptor agonist systems were studied: 1) estrogen receptor (ER) agonists of the SERM family because of the central role estrogen plays in the maintenance of bone mass in women and men, 2) vitamin D receptor (VDR) agonists were studied because of known alterations in vitamin D levels during spaceflight, and 3) a new class of receptor, the calcium sensing receptor (CaR) was studied because of the potential importance of this receptor in modulating bone cell differentiation and function. The aim was to determine if the separate and interactive response of bone cells would promote osteoblastic activity and decrease osteoclastic differentiation thus maintaining bone formation in excess of bone resorption. Both in vitro and in vivo studies have been accomplished.
Studies with the SERMs idoxifene and raloxifene have identified osteoblastic patterns of gene expression that are distinct for each of the agonists. A potentially effective countermeasure to vitamin D deficiency, the analogue EB 1089, which may also modulate cell differentiation has been defined. Multiple osteoblastic cell strains, have been shown to express the CaR opening the possibility for regulation of osteoblast/osteoclast differentiation using CaR receptor agonists.
Interaction between receptor agonists has been investigated. Studies in the hindlimb suspended rat are in progress with the goal of in-flight testing. Thus, the overarching result of these studies rests not only in the definition of specific receptor responses to a series of agonists, but in the demonstration that the agonists interact with each of the individual receptors to promote osteoblastic activity and decrease osteoclastic activity. It is this synergy that will direct the future development of novel bone active agents.

Research Results (O'Malley et al):

1. CaR is expressed in most osteoblasts in sections of bovine, murine and rat

activity leading to bone resorption. The genetic expression regulating these modulators is under study in the laboratories of L Suva and R. Turner.

Research Results:

1. Hindlimb unloading, within 10 minutes, diminishes blood flow to the femoral and tibial methaphysis (trabecular bone), diaphysis (cortical bone) and marrow. Prolonged unloading, e.g., over 7 days in the hindlimb suspended preparation, further decreases perfusion of the femoral shaft and marrow. The prolonged decrease in blood flow to the marrow space is of interest in view of changes in marrow osteoeprogenitor cells after unloading..

2. The decline in blood flow to the hindlimb coincides with a diminished mineral apposition rate, diminished bone density and decreased mass of both cortical and trabecular bone. Correspondingly, increased blood flow and fluid shift cephalad coinides with reported increase in bone mass in the upper trunk and head following hindlimb suspension.

3. Gene expression patterns: In collaboration with Drs. Suva and Turner preliminary studies have demonstrated significant differences in gene expression following hindlimb suspension measured by gene array methods.

4. The countermeasure actions of β-agonist agents is currently under study. Determination of the time course of blood flow changes indicates that skeletal blood flow capacity during standing is diminished with 28 days of hindlimb standing: e.g., skeletal unloading alters the structure of osseous circulation. This raises the question of post-microgravity ischemia, and may also relate to the significant delay experienced in reconstituting normal bone mass after flight.

This study emphasizes the important role played by altered bone circulation during exposure to microgravity conditions. Studies of bone cell gene expression and cytokine production from marrow and vascular endothelial cells, now topics of great interest to the vascular physiologist finds ready application to the problem of bone loss. The definition of countermeasures for this problem requires additional investigation. Negative pressure lower body instrumentation may not provide the answer. Administration of various vasoactive agonist agents may also not be feasible because of the systemic effects of these agents. Nevertheless, maintenance of lower extremity blood flow is essential to the maintenance of bone/muscle health.

Effect of Graded Mechanical Loading and Ibandronate: in the Adult Rat Model of Microgravity (Schultheis et al)

Exercise programs employed during spaceflight over the past 30 years have failed to prevent either muscle or bone loss. As efforts are directed to 6 month flights in the ISS or possible 3 years exploration to Mars, there is increased emphasis on the development of effective countermeasures. Several laboratories have proposed mechanical systems to limit the detrimental effects of microgravity on the musculoskeletal system. These include resistive exercise, methods for providing the pull of artificial gravity in the spacecraft and mechanical loading systems. Of these, positive tissue responses have been reported after the application of mechanical strain induced by repetitive vibrational stimulation or impact stimulation of the lower extremities. Loading by partial weight bearing to simulate Mars gravity and added vibrational strain has been evaluated using a novel force plate with the hindlimb suspended rat. The effect of a simultaneously administered potent bisphosphonate, ibandronate (Roche), on bone density and other related parameters has been determined in this system.

Research Results

1. Using 3 mo old female rats, hindlimb suspension with 50% weight bearing on the forelimbs failed to maintain normal cortical or trabecular bone mass in the forelimb as compared with free-roaming controls. Partial weightbearing however, maintained bone formation rate and extrinsic and intrinsic (material) mechanical properties. Partial weightbearing preserved geometrical properties of cortical bone in 5 mo old animals.

2. Dynamic loading at 3 Hz which lies in the normal ambulatory spectrum of the rat superimposed on partial (50%) weightbearing was effective in preserving cortical bone area, polar moment of inertia and section modulus. Bone formation rate was maintained under these conditions. However, trabecular bone density and the stiffness of bone were not maintained.

3. Ibandronate treatment retarded the decrease in trabecular density and increased bone formation rate. Ibandronate increased trabecular bone density in free-roaming animals. Significant increases in structural properties of cortical bone were observed with ibandronate under conditions of partial weightbearing.

4. Biochemical analyses of bone matrix proteins in suspended animals demonstrates a decrease in collagen (ug/mg bone) and proteoglycan (ug/mg bone) compared to free-roaming controls, but no change in osteocalcin content. Ibandronate treatment increased collagen content but not proteoglycan in controls and suspended animals

These results suggest that a combination of mechanical loading and pharmacologic inhibition of bone resorption with ibandronate, a potent third generation bisphosphonate, may provide optimal protection against bone loss in humans exposed to microgravity. Ibandronate may also have significant effects on matrix protein content.

Skeletal Structural Consequences Of Reduced Gravity Environments (Ruff et al.)

Protection against the risk of fracture while in microgravity environments for extended periods of time requires an understanding of the mechanisms involved in bone loss as well as the means to monitor sequential changes in bone mass. The primary goal of this project was to document changes in mechanically relevant geometric parameters of bone structure in humans and in animal models under microgravity conditions, during altered mechanical loading on the forelimb in hindlimb suspended rats, and during treatment with ibandronate, an antiresorptive bisphosphonate agent (see Schultheis). Three data sets were analyzed: 1) DEXA data derived from pre- and post studies of Mir cosmonauts, 2) DEXA data from human subjects at prolonged bedrest(Dr. Adrian LeBlanc, PI) , and, 3) pQCT measurements of bone mass parameters in rats subjected to 35 days of hindlimb suspension and treatment with ibandronate (Dr. L. Schultheis, PI) . Changes in hip fracture risk due to prolonged spaceflight were estimated using data derived from a 3 D finite element analysis of the femur and a dynamic loading model.

Research Results:

1. 112 day bedrest leads to a decline in BMD and geometric section properties. Hip DXA analysis in control subjects before and after 17 weeks of bedrest shows a significant decline in femoral neck bending strength, as much as 2% /month. However, ranges of responses varied from an 8% decline to a 1% gain, highlighting not only the importance of consistency in experimental conditions, but also the wide variation in individual response to conditions of unloading. This variation in response, now demonstrated by geometric/structural analysis was also seen in BMD measurements following flight.

2. Hip DXA scans on Mir cosmonauts in collaboration with Dr. LeBlanc et al., studies after an average of 178 days in flight again showed a decline in bone section modulus of more than 1% per month in the femur neck. In contrast to structural changes associated with normal aging or in bed rest subjects, periosteal diameter did not increase at any proximal femur location. This absence of periosteal expansion in association with endocortical bone resorption thus increases fracture risk. Failure to increase the expression of genes related to local grwoth has been reported in the periosteal layer of rodents following flight by Turner et al.

3. 3-D finite element analysis based on DXA data derived from cadaveric specimens estimates fracture associated with falls after bone loss after 12 months of spaceflight. The initial factors of risk for fracture in a midstance loading configuration were 0.62 for males, and 0.71 for females representing an increase in factors of risk by 26%. This would apply to risk following a traumatic event or that during work in a hazardous environment. Quantitatively similar results were obtained using 2-D curved beam theory analysis of DXA data. The Technology Team (Drs. Charles, Beck and Pisacane) could obtain such measurements during flight in with the AMPDXA bone densitometer scanning instrument under construction at the Hopkins Applied Physics Laboratory.

4. pQCT measurements in the hindlimb-suspended rat model of microgravity (see Dr. Schuletheis' report for full data analysis) indicate that partial weight bearing conditions (50% forelimb weight bearing) may not suffice to maintain normal bone remodeling. Mechanical stimulation through forceplate vibration may maintain cortical, but not trabecular , bone mass. The administration of an antiresorptive bisphosphonate, ibandronate, does prevent bone loss under these conditions. The results suggest that combined mechanical loading plus a pharmacological intervention may be the optimal method for reducing bone loss under conditions of prolonged microgravity exposure.

II. RISK REDUCTION ACHIEVED BY PROGRAM

The research results presented above and in the Principal Investigators reports, are targeted at risk reduction through the development of specific countermeasures to bone loss. Effective mechanical loading which may maintain both muscle and bone mass is critical in this regard, 50% weight-bearing may not be effective in reducing bone loss. Mechanical stimulation by vibration reported here is also under study in other laboratories. Although optimal levels of vibrational or impact stimulation have yet to be defined, and the instrument by which this can be applied in the flight setting has yet to be designed., the results clearly point to vibrational systems as potentially important countermeasure in the effort to decrease bone loss.

An understanding of the relationship of bone loss to altered vascular responses during weightlessness is critical to effective countermeasure development. Current studied are aimed at the pharmacological and biomechanical methods for maintaining blood flow at a specific site in the lower trunk. . Here, risk reduction depends on progress in this area.

Two classes of antiresorptive drugs have been studied in these projects, and on-going research is being pursued in the hind-limb suspended rat model. Bisphosphonate drugs clearly may be useful in mitigating bone loss associated with microgravity exposure experienced by humans. Correction of abnormal vitamin D metabolism due to microgravity appears possible through the use of newer vitamin receptor agonists. The results suggest that manipulation of the vitamin D receptor and estrogen receptor via the non-calcemic analog EB1089 and the bone selective ER agonist raloxifene appear to have potential in counteracting unloading-induced bone loss. . As yet less defined, but approaching the level of animal experimentation are CaR agonists which probably will be effective in the regulation (positive and negative) of progenitor cell differentiation, both for osteoblasts and osteoclasts.

Furthermore, these reports extend the potential for a real-time assessment of fracture risk under conditions of prolonged spaceflight at 0 gravity and during Mars habitation at 0.38 X g. The studies reported here point to the necessity of developing a flight-compatible means for sequential measurements of bone density and bone structural properties during flight and during extraterrestrial habitation. As demonstrated in earth studies and in the Mir cosmonauts, the

application of analyses reported by Dr. Ruff and his colleagues holds promise for the real-time assessment of countermeasures effectiveness. They afford the ability to modify activity levels, exercise programs or medication regimen to maintain optimal bone strength in each individual.

III PROGRAMATIC IMPLICATIONS OF RESEARCH RESULTS

A major implication of these results is that the response of bone to microgravity probably involves mechanisms that are not fully explained by known alterations in traditional calcitropic hormones or mineral balance. Furthermore, the results suggest, pending additional research addressing the issue of mechanical loading, that *both* mechanical loading and pharmacological suppression of bone resorption will be important for the maintenance of bone mass during prolonged microgravity exposure.

Key issues standout as important foci for continued research:

1. The first NSBRI program did not permit addressing important critical path risks: fracture healing, soft tissue injury and renal stone formation. In addition, the interplay between muscle and bone has not been included in the projects initially funded. These topics will be addressed during the second three year cycle of funding.

2. The investigators on the Bone Team have gained a remarkable level of familiarity with elements of space physiology related to bone loss that are critical to the maintenance of a healthy crew during prolonged spaceflight. This expertise is at a point where closer working relationships with NASA scientists would enhance opportunities to move from ground to flight testing.

3. The results reported here emphasize the need to focus on countermeasure development. They illustrate the need to selectively encourage basic science, while at the same time, moving towards flight testing. .This is evident in receptor agonist studies which have progressed from cellular investigations to animal testing, to the use of a novel intravenous bisphosphonate in rodents to testing these agents in humans in a chronic bed rest setting, and to definition of effective rates of vibrational stimulation pending the design of a flight-testable instrument. Similarly, collaborative investigation on bone structural characteristics and fracture risk can be continued in flight using a new and flight compatible bone density device under development by the Technology team..

Novel Receptor-Based Countermeasures to Microgravity-Induced Bone Loss

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2. Executive Summary

The biological actions mediated by the estrogen receptor (ER), vitamin D receptor (VDR) and extracellular Ca^{2+}_o-sensing receptor (CaR) play key roles in the normal control of bone growth and skeletal turnover that is necessary for skeletal health. These receptors act by controlling the differentiation and/or function of osteoblasts and osteoclasts, and other cell types within the bone and bone marrow microenvironment. The appropriate use of selective ER modulators (SERMs) which target bone, vitamin D analogs that favor bone formation over resorption, and CaR agonists that may both stimulate osteoblastogenesis and inhibit osteoclastogenesis as well as the function of mature osteoclasts, should make it possible to prevent the reduction in bone formation and increase in bone resorption that normally contribute to the bone loss induced by weightlessness. Indeed, there may be synergistic interactions among these receptors that enhance the actions of any one used alone. Therefore, we proposed to: 1) assess the *in vitro* ability of novel ER, VDR and CaR agonists, alone or in combination, to modulate osteoblastogenesis and mature osteoblast function under conditions of 1g and simulated microgravity; 2) assess the *in vitro* ability of novel ER, VDR and CaR agonists, alone or in combination, to modulate osteoclastogenesis and bone resorption under conditions of 1g and simulated microgravity; and 3) carry out baseline studies on the skeletal localization of the CaR in normal rat bone as well as the *in vivo* actions of our novel ER- and VDR-based therapeutics in the rat in preparation for their use, alone or in combination, in well-established ground-based models of microgravity and eventually in space flight.

We have found that the CaR is expressed in osteoblastic cells as well as in bovine, murine and rat bone and that activation of this receptor in osteoblastic cells leads to activation of an outward K^+ channel and chemotaxis of calvarial osteoblasts in response to elevated Ca^{2+}_o. The CaR is also present in osteoclast precursors and in osteoclast-like cells formed *in vitro* and plays roles in regulating osteoclastogenesis and osteoclast chemotaxis. In our VDR studies, we have examined the ability of the VDR agonist, EB1089, which is less calcemic than calcitriol, to regulate osteoblastic gene expression and find that it is more potent than calcitriol. In addition, gene expression in the MG-63 osteoblastic cell line was characterized in the Slow Turning Lateral Vessel (STLV) culture system, which approximates many aspects of microgravity. Many genes were down-regulated in comparison to monolayer cultures grown at unit gravity, and responses to VDR agonists were less robust. In ongoing hindlimb suspension studies in male rats, EB1089 was able to prevent unloading-induced bone loss measured at the proximal tibia, while calcitriol was able to increase bone mineral density. However, increases in serum calcium in calcitriol-treated animals, not observed in EB1089-treated rats, indicate that the latter is a superior countermeasure. EB1089 is also a less potent stimulator of osteoclast formation in comparison to calcitriol. Finally, our ER studies have revealed that osteoblastic gene expression patterns induced by estradiol and the SERMs idoxifene and raloxifene, are distinct even though all agents are capable of inhibiting bone loss due to sex steroid depletion. Raloxifene does not reduce bone mineral density in normal female rats and has only modest effects on biochemical markers of bone turnover suggesting its use in gonad-intact populations should not increase the risk of bone loss via inhibiting endogenous estrogens. In ongoing hindlimb suspension studies in ovariectomized female rats, raloxifene and estradiol, individually, appear able to prevent loss of bone mineral density. Since raloxifene does not exert undesirable, estrogen-like effects in reproductive tissues, it has the potential to be an acceptable countermeasure to disuse-induced bone loss. Ongoing studies will continue to examine the use of EB1089 and raloxifene, alone

and in combination, to prevent bone loss in male and female rats induced by hindlimb suspension.

Collectively these studies suggest that manipulation of VDR and ER activity has the potential to reduce the risk of bone loss resulting from the microgravity environment encountered during Space travel. Importantly, our data also suggest that novel ligands for these two receptors that significantly attenuate the negative side effects of the natural ligands can be effectively employed to reduce unloading-induced bone loss, and the ensuing risks of bone fracture.

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4. Project Research Activity

4A. Original Hypothesis

The advent of manned space flight has opened new frontiers for human exploration. However, humans living in a microgravity environment for an extended period of time experience numerous physiological changes, one of these being a reduction in bone mass. It is well established that weight-bearing exercise significantly contributes to a healthy skeleton, and it is highly likely that the lack of mechanical loads on the musculoskeletal system is one of the primary causes of microgravity-induce osteopenia. However, changes in the concentration of systemic factors that regulate processes of bone formation and resorption [growth factors, calcium, steroids and 1,25-dihydroxyvitamin D] have been noted in humans and animals living in a microgravity environment, and it is probable that these alterations also negatively influence bone. The biological actions of these hormones are mediated by their respective receptors [estrogen receptor (ER), vitamin D receptor (VDR) and extracellular Ca^{2+}_o-sensing receptor (CaR)] which play key roles in the normal control of bone growth and turnover that are necessary for skeletal health. These receptors act by controlling: (1) the differentiation of the precursors of osteoblasts (OBs) and osteoclasts (OCs), (2) the functions of mature OBs and OCs and/or (3) other cell types within the bone and bone marrow microenvironment that control (1) and (2) (*e.g.* stromal cells and monocytes). Therefore, it is our hypothesis that the appropriate use of novel ER-, VDR- and CaR-based therapeutics will make it possible to prevent the reduction in bone formation and increase in bone resorption that normally contribute to the bone loss induced by weightlessness. Indeed, there may be synergistic interactions among these receptors that enhance the actions of any one used alone.

4B. Original Objectives

Since skeletal strength is essential upon return to a gravity environment, be that Earth or Mars, it is necessary to establish methods that can maintain sufficient bone mass to enable humans to work without increased risk of fracture. Resistive exercise has been used with partial success to maintain bone mineral density during microgravity, but decreases in bone formation and/or increases in bone resorption still occur and are of sufficient magnitude to seriously compromise skeletal integrity and, therefore, it is necessary to devise additional, more effective countermeasures. Since systemic factors, such as serum calcium, vitamin D and estrogens, play important roles in the regulation of bone formation and maintenance, it is possible that modulation of the activities of the receptors for these agents may provide a mechanism to counteract the deleterious effects of microgravity. Recently, several pharmacological agents have been developed that have the potential to positively regulate bone mineralization, and it is our overall goal to use these novel estrogen, vitamin D and Ca^{2+}_o-sensing receptor-based therapeutics to prevent the loss of bone mineral density that normally occurs during prolonged space flight. To begin to assess the ability of these compounds to regulate bone cell differentiation and function, we proposed to examine their ability to modulate estrogen receptor, vitamin D receptor, and Ca^{2+}_o-sensing receptor-mediated actions in the three specific aims outlined below.

4C. Original Specific Aims

1. To assess the *in vitro* ability of novel receptor agonists of the ER, VDR and CaR, alone or in combination, to modulate osteoblastogenesis and mature osteoblast function under conditions of 1g and simulated microgravity.
2. To assess the *in vitro* ability of novel receptor agonists of the ER, VDR and CaR, alone or in combination, to modulate osteoclastogenesis and bone resorption under conditions of 1g and simulated microgravity.
3. To examine the skeletal localization of the CaR in normal rat bone as well as the *in vivo* actions of our novel ER- and VDR-based therapeutics in the rat in preparation for their use, alone or in combination with CaR-based therapeutics, in established models of microgravity and eventually in space flight.

4D. Summary of Year 1 Progress

4Di. Aim 1

In the first year of this project, we investigated whether the CaR is expressed in various osteoblastic bone cell lines. These studies were done as a foundation for *in vitro* studies in subsequent years addressing the impact of CaR agonists, with or without VDR and ER agonists on the function of osteoblasts in vitro. We used reverse transcription-polymerase chain reaction (RT-PCR) and Northern analysis to demonstrate the expression of CaR transcripts as well as immunocytochemistry and Western analysis with CaR-specific antisera to document the presence of CaR protein in the following osteoblast-like cell lines -- murine MC3T3-E1 osteoblastic cells, rat UMR-106 cells, and human SAOS-2 and MG-63 cells. Furthermore, raising the extracellular calcium concentration (Ca^{2+}_o) stimulated the proliferation and induced chemotaxis of MC3T3-E1 cells, previously described actions of Ca^{2+}_o on these cells that may be important in mobilizing osteoblastic precursors to sites of recent bone resorption. It is possible, therefore, that the CaR mediates some or all of the known effects of Ca^{2+}_o on osteoblastic function.

We also used the same four techniques to document expression of CaR mRNA and protein in the ST-2 mouse stromal cell line. Stromal cells could either communicate with osteoblasts via paracrine or direct physical interactions or possibly serve as osteoblast precursors derived from the mesenchymal stem cell population that differentiates into osteoblasts, chondrocytes, adipocytes, fibroblasts or myocytes. As with MC3T3-E1 cells, high Ca^{2+}_o stimulated both the proliferation and chemotaxis of ST-2 stromal cells -- actions that could participate in the osteoblastic recruitment needed for replacing bone resorbed during the osteoclastic phase of bone turnover.

Both estrogens and vitamin D modulate osteoblast function and/or differentiation, and it was one of our overall goals to identify compounds that will be more beneficial than endogenous ER and VDR ligands with respect to potency and/or tissue specificity. In order to begin these studies, we have characterized VDR, ERα and ERβ responses in the osteoblastic-like cell line, MG-63, using both osteocalcin secretion and synthetic reporter gene assays as end points. The original plan to use MG-63 and ROS17/2.8 cells to analyze responses of osteoblast-like cells to

VDR and ERα/β agonists was expanded to include the MC3T3-E1 line because it is one of our goals to assess VDR, ER and CaR agonists in combination, and our first year studies (see above) demonstrated CaR expression in these cells. Initially, MG-63 cells were our primary focus because this cell line can be used to validate that the STLV bioreactors mimic the effects of microgravity on osteoblastic cells. Previously, other investigators had analyzed the effect of microgravity on MG-63 cells by measuring several cellular markers in cells cultured in a microgravity environment (aboard the Foton-10 satellite) *versus* cells maintained at unit gravity (Carmeliet et al, J. Bone Mineral Research 12(5):786-794, 1997). Our bioreactor studies are described below. With respect to VDR responses, it was our plan to measure both established markers of 1,25D action in bone cells, such as osteocalcin production which depends upon 1,25D and other factors, as well as the activities of transfected reporter plasmids to obtain a direct measurement of receptor function only. The measurement of osteocalcin by ELISA assay as a function of VDR agonist concentration is particularly important since this is one of the biomarkers we will assess in our STLV bioreactor microgravity model system. Similarly, we have measured the ability of ERα and ERβ to activate transcription of several transfected reporter plasmids as a function of estradiol and selective ER modulator (SERM) concentration. In addition, we have assessed the influence of estradiol on vitamin D-induced osteocalcin expression.

Our initial studies showed that bone cells could not be transfected with high efficiency using conventional, lipid-based transfection methods such as Lipofectamine or Lipofectin. Since our goal was to measure the responses of endogenous receptors if at all possible, it is critical that we use a high efficiency transfection procedure. We adapted our lysine-coupled adenovirus transfection procedure for this purpose. Briefly, carrier adenovirus is prepared by UV irradiating replication deficient adenovirus and covalently coupling polylysine to the adenovirus. This reagent can be stored frozen until needed. Reporter plasmids are ionically bound to the polylysine, additional polylysine is added and the coupled adenovirus added to cells. By modifying virus/cell ratios we can deliver either chloramphenicol acetyltransferase or luciferase based reporters as well as receptor expression plasmids, as required.

Using this adenovirus transfection technique, we have shown that we can readily detect the activity of the endogenous VDR in MG-63, ROS 17/2.8 and MC3T3-E1 cell lines. This offers an advantage over exogenous receptor in that we will be able to measure changes in receptor expression as well as function as a result of microgravity. We have done hormone titration curves with 1,25-dihydroxyvitamin D3 (1,25D) and the less calcemic analog EB1089 from Leo Pharmaceuticals using osteocalcin-based reporters as well as synthetic reporters. We have found that EB1089 is both more potent and efficacious than 1,25D in inducing osteocalcin expression. One nM EB1089 was required to induce maximal levels of osteocalcin whereas 100 nM 1,25-D was required. Inductions were 50-100 fold. Under these conditions we also can detect the activity of the endogenous VDR using either CAT or luciferase reporters. In each case we can measure hormone-dependent inductions of 10-20 fold. Using a VDREtkCAT reporter, which contains a vitamin D responsive element, we found good induction with EB1089. In each case, EB1089 is more potent than 1,25D. Because VDR acts as a heterodimer with retinoid X receptor (RXR), we measured the effect of its ligand, 9-cis retinoic acid on VDR function. Even at suboptimal doses of VDR agonist, 9-cis retinoic acid had no effect. This is consistent with a number of reports suggesting that, in most cases, RXR ligands do not alter VDR function.

Finally, at the level of reporter activation, we see no effects of estradiol on VDR function. However, we expect that more complex biological actions will be affected by estradiol. Some of these responses were also tested in the STLV bioreactor (see below).

Using synthetic target genes containing one or three copies of an estrogen response element linked to a minimal promoter and the luciferase target gene, we assessed endogenous ER activity in MG-63 cells. No activation of target gene expression was detected in cells treated with either estradiol or 4-hydroxytamoxifen. However, when ERα was expressed in this cell line via a constitutively active mammalian expression vector, luciferase gene expression was increased 3- to 12-fold, depending on the target gene examined (one ERE *versus* three EREs, respectively). Taken together, these data suggest that there are insufficient levels of functional ER in MG-63 cells to mediate transcriptional activity, and that this apparent insensitivity can be overcome by expressing exogenous ERα. Based on other investigator's results showing ERα mRNA expression in MG-63 cells (Mahonen & Mäenpää, Biochem Biophys Res Comm 205(2):1179-1186, 1994), we had anticipated being able to measure endogenous ER activity. However, our Western blot analysis using an ERα-specific monoclonal antibody indicates that these cells have undetectable amounts of ERα protein, which is consistent with the results of our transcriptional experiments. We have also introduced ERβ into these cells via expression vector, and can upregulate the activity of this receptor ~3-fold with estradiol. For both ERα and ERβ, estradiol stimulates reporter gene expression in a dose-dependent fashion, with maximal stimulation occurring at 10 nM estradiol. In contrast, the partial ER agonist, 4-hydroxytamoxifen, appears to preferentially stimulate the transcriptional activity of ERα, while not affecting ERβ function. Interestingly, the effect of this moderately selective ER agonist does not appear to be dose-dependent, and increases the activity of the 1xERE-TAT-Luc target gene by ~1.5-2 fold. We have also assessed the ability of ER agonists to modulate osteocalcin in this cell line, and have found that, as expected, estradiol alone was unable to stimulate osteocalcin secretion.

The ability of estradiol and various SERMs were assessed in the other two osteoblastic cell lines, ROS17/2.8 and MC3T3-E1. The results of the experiments indicated that there was insufficient ER expression in ROS 17/2.8 cells to measure the transcriptional activity of endogenous ERs, even with reporter genes delivered by our adenovirus methodology. Western blot analysis also failed to detect ERα expression in these cells. While ER expression in ROS 17/2.8 cells has been reported (Migliaccio et al, Endocrinology 130(5): 2617-2624, 1992), the level is extremely low (200-500 binding sites/cell). We therefore concentrated our studies on estrogen receptor activity in MC3T3-E1 cells. Our initial synthetic target gene expression experiments indicate that these cells express functional ERs. We are currently expanding these studies to examine the effectiveness of SERMs in these experiments, and characterizing ERα/ERβ protein expression. As stated above, it is one of our objectives to assess the combined activities of VDR, CaR and ER agonists, and studies of the expression of ER activity in these cells lines are an important step towards achieving this goal.

Upon obtaining funding for this project, we had four STLV bioreactors manufactured, and have been successful in our initial attempts to grow MG-63 cells in these devices. In simple terms, the bioreactors consist of cylindrical culture vessels that rotate around their central core, such that the centrifugal force generated by rotation negates normal gravitational effects. Cells grown on microcarrier beads (Cytodex 1) in the bioreactors take on an altered morphology in

comparison to those grown on microcarrier beads in petri dishes at unit gravity, indicating that the cells are responding to their altered environment. An important component of our experiments is to validate these bioreactors as a model of microgravity. Therefore, we are presently assessing the ability of these cells to secrete osteocalcin in response to vitamin D treatment, relative to cells grown at unit g. Based on previous work, we anticipate that vitamin D induction of osteocalcin secretion should be very low in comparison to that observed when cells are grown at unit gravity.

4Dii. Aim 2

As an approach to studying the potential role of the CaR in modulating the functions of cells of the osteoclast lineage, we initially examined the mouse monocytic cell line, J774, and human peripheral blood monocytes for expression of the CaR. Cells of the macrophage/monocyte lineage serve as precursors for the formation of osteoclasts through a process involving cell differentiation and eventual fusion to form multinucleated bone-resorbing cells. Moreover, high Ca^{2+}_o is known to stimulate the production of the osteotropic cytokine IL-6 from peripheral blood monocytes *in vivo* and *in vitro*. In our first year studies, we have found that most (80-85%) peripheral blood monocytes express abundant CaR protein and readily detectable mRNA for the receptor, as does the J774 monocytic cell line. In addition, we showed that the myelomonocytic leukemic cell line, HL-60, upregulates CaR protein expression when differentiated along the monocyte/macrophage pathway by addition of 1,25-dihydroxyvitamin D or the phorbol ester, PMA. Indeed, monocytes formed from 1,25-dihydroxyvitamin D-differentiated HL-60 cells are known to form multinucleated, osteoclast-like cells capable of resorbing bone, suggesting that such monocyte-differentiated HL-60 cells could be a useful model for the process of osteoclastogenesis *in vitro* and the putative role of the CaR in this process. Finally, mouse marrow cultures can be induced to form osteoclasts *in vitro* by the addition of 1,25-dihydroxyvitamin D. When we examined such cultures for expression of the CaR during *in vitro* osteoclastogenesis, we found CaR expression both by both the mononuclear cells that formed aggregates and then fused to form multinucleated, osteoclast-like cells expressing tartrate-resistant acid phosphatase and calcitonin receptors (osteoclastic markers) as well as the multinucleated cells themselves. Thus the CaR may be present in osteoclast precursors and mature osteoclasts and could potentially mediate the inhibitory actions of Ca^{2+}_o on osteoclast function that could provide an effective target for CaR-active agents in the prevention of microgravity-induced bone loss.

4Diii. Aim 3

As part of our collaboration with Dr. Larry Suva, we have obtained frozen sections of rat tibia in order to investigate whether *bona fide* osteoblasts and osteoclasts in authentic bone express the CaR. In addition, we carried out preliminary immunocytochemical studies with anti-CaR antiserum on sections provided to us by Dr. Steven Goldring of the Beth Israel-Deaconess Medical Center in Boston of a giant cell tumor of bone and the bone adjacent to an inflamed joint of a patient with rheumatoid arthritis. In both cases, we observed that a substantial fraction of multinucleated, osteoclast-like cells stained positively for the CaR. These results suggest that at least some osteoclast-like cells formed *in vitro* as well as those present in bone *in vivo* express the CaR and could potentially mediate the known inhibitory actions of Ca^{2+}_o on osteoclast

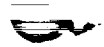
function that could make CaR agonists of possible therapeutic utility, alone or in combination with agonists of the VDR and ER, in preventing microgravity-induced bone loss.

4E. Summary of Year 2 Progress

This portion of our report outlines the results of the second year of studies designed to investigate the comparative ability of ER, VDR and CaR agonists to regulate osteoblastogenesis, osteoblast function and osteoclastogenesis. We have continued our characterization of the expression of the CaR in normal bone and bone cell lines, and used our established conditions for transfecting osteoblast-like cell lines to continue measurement of the activation of the VDR and ERs using various promoters as a function of natural and synthetic ligands, alone or in combination with other activators. Although we have been able to detect the activity of the endogenous VDR and ER using transient transfection of reporter constructs, in the past year we have focussed on establishing assays for natural biological responses. These included alkaline phosphatase activity, osteocalcin secretion, vitamin D receptor levels, and measurement of mRNA for target genes such as collagen Iα1 and osteocalcin. We proposed to compare the effect of selected VDR and ER agonists on induction of these targets. In addition to studies performed under standard cell culture conditions (*e.g.* monolayer growth conditions at 1g), we have done experiments in four Slow Turning Lateral Vessel (STLV) bioreactors. These bioreactors have enabled us to assess the effect of simulated microgravity on the ability of cells to respond to our proposed hormonal countermeasures. We planned to standardize the STLV model, compare the results with those of MG-63 cells grown aboard the Foton 10 satellite, and to use this model to compare responses of cells to calcitriol and synthetic VDR agonists. The results of these studies provide a foundation for future experiments that will examine the ability of the most promising pharmacological countermeasure(s) to maintain bone mineral density and strength in the hindlimb suspended rat, an established model of microgravity-induced osteopenia.

4Ei. Aim 1

During the first half of Year 2, we have continued to investigate the CaR's presence in various osteoblastic bone cell lines as well as in primary osteoblasts and their precursors. These studies are necessary to serve as a foundation for *in vitro* and *in vivo* studies addressing the impact of CaR agonists, with or without VDR and ER agonists, on osteoblastogenesis and the function of mature osteoblasts. We have used RT-PCR and Northern analysis to detect CaR transcripts as well immunocytochemistry and Western analysis with CaR-specific antisera to identify CaR protein in the following osteoblast-like cell lines, as noted above--murine MC3T3-E1 osteoblastic cells, rat UMR-106 cells, and human SAOS-2 and MG-63 cells, as well as in primary murine calvarial osteoblasts. All of these osteoblastic cells express CaR transcripts and/or protein. During the past several months, we have also initiated a collaborative study with Dr. Keith Hruska in St. Louis directed at examining the presence and role of the receptor in the development of human osteoblasts from their mesenchymal precursors in a primary culture model that he has developed. In those studies, we have shown that the precursor cells as well as more mature osteoblasts induced to differentiate using an osteogenic medium express the CaR as assessed by immunocytochemistry using a specific, polyclonal anti-CaR antiserum. Thus it is possible that the CaR mediates some or potentially all of the known effects of Ca^{2+}_o on







osteoblastic function-- including effects on both osteoclastogenesis and the function of mature OBs--although pharmacological studies from other investigators support the presence of additional calcium-sensing mechanism(s) in cells of the osteoblastic lineage.

We have also used the same four techniques to show that CaR mRNA and protein are expressed in the ST-2 mouse stromal cell line, as described above. Since the actions of elevated levels of Ca^{2+}_o on both osteoblasts and stromal cells could promote increased bone formation, it will be important to prove in future studies that these actions are CaR-mediated as a basis for the possible use of CaR agonists, alone or in combination with VDR and ER agonists, as a countermeasure against microgravity-induced bone loss.

Estrogens modulate the function and/or differentiation of osteoblasts, and it is one of our overall goals to develop the use of selective estrogen receptor modulators that will function as ER agonists in bone but not in reproductive tissues. In our first year's studies, we concentrated on the use of a human osteoblastic cell line, MG-63, and used this to screen various SERMs for estrogen agonist-like activity in transient transfection assays. These cells did not contain sufficient levels of ER to mediate this response, and in order to assess agonist activity we had to introduce an ER expression vector. In this year's studies, we have utilized a cell line, MC3T3-E1, which expresses sufficient ER levels to enable reporter gene expression to be measured without the addition of exogenous ER. This has enabled us to examine the effects of various ER ligands on osteoblastic cell function without possible artifacts that may arise from overexpressing ER, and obviates the need to create stable cell lines. We have attempted by Western blot analysis to determine whether ERα or ERβ is expressed in these cells, but our antibodies are not sensitive enough to detect the expression of either protein, although they do detect expression in several positive control cells. Using an RT-PCR approach we have detected mRNA for ERβ. Previously it was demonstrated that these cells express ERα mRNAs (J. Bone & Mineral Research 8:1103-1109, 1993). Thus, MC3T3-E1 cells appear to expression both ER isoforms. This finding parallels published data that indicates that bone and several bone cell lines express mRNAs for both ERα and ERβ.

We have gone on to use this cell line to examine the ability of various SERMs to activate the expression of a target gene introduced via our established transient transfection methodology. Using a target gene containing 3 copies of an estrogen response element (ERE) linked to a minimal promoter and luciferase target gene, we have been able to assess the ability of various SERMs (idoxifene, 4-hydroxytamoxifen, EM-01538, GW5648 and ICI 182,780) for their transcriptional activity in comparison to estradiol. Estradiol is a full agonist in this system and stimulates the activity of the reporter gene by approximately 4.0-fold. Of the tested SERMs, Idoxifene stimulates activity 2.3-fold, 4-hydroxytamoxifen 1.5-fold, EM-01538 1.8-fold, and ICI 182,780 by 1.8-fold. All compounds activated the target gene in a dose-dependent manner. These experiments indicate that these SERMs will act as ER agonists on a classical ERE in reporter gene assays. At that time, and based on these assays, the SERM with the best agonist activity appeared to be idoxifene.

At the present time, it is not clear if all the effects of estrogens in bone are mediated via the ER binding to an ERE target gene and activating transcription, and it is possible that estrogens binding to the ER may work via binding to and regulating the activity of other transcription factors such as AP1, Sp1, NFκB, C/EBPβ or other unidentified mechanisms. Therefore, while reporter gene assays are useful to determine the relative ability of ligands to

regulate the activity of the ER itself, we have performed studies to examine the ability of SERMs and estradiol to induce expression of various mRNAs or proteins characteristic of osteoblasts so their effects can be assessed in more complex systems (see below).

With respect to vitamin D receptor studies, the majority of the transient transfection studies had been completed in year 1, and it was our goal to proceed to establish assays for natural biological responses. Therefore, we have established and standardized assays for intracellular alkaline phosphatase activity, osteocalcin secretion, vitamin D receptor expression using immunoblotting, and measurement of target mRNAs including collagen Iα1, osteocalcin, and osteonectin. We had originally planned to use Northern analysis to measure the mRNA levels, but the quantity of material in some of the experiments was insufficient. Therefore, we have established RNAse protection assays (RPA) to compare message levels. In each of the assays described above, EB1089 was more potent (10-100 fold) in inducing the target in MG-63 cells than was calcitriol. Moreover, in some cases such as VDR expression, EB1089 also induced higher levels of the target.

In addition to the classical nuclear receptor mediated actions of vitamin D, there are numerous reports of rapid membrane effects of the active metabolite of vitamin D, calcitriol, that induce protein kinase C activity and may be important for the overall biological actions of calcitriol. We have found that calcitriol rapidly (within minutes) induces activation of mitogen activated protein kinase (MAPK) and that this activation is sustained for at least two days. Because MAPK can be activated through protein kinase C activation, we have used this as an assay for rapid effects of vitamin D receptor agonists. EB1089 is more potent and more effective in inducing this response, suggesting that it is an appropriate agonist for the rapid responses as well as classical transcriptional responses. The responses of MG-63 cells to the novel non-secosteroid vitamin D receptor agonist from Ligand Pharmaceuticals are currently being measured and these studies will be completed by the end of year 2. Preliminary studies assessing the effects of calcium levels on the response to VDR agonists and the functional interactions between CaR and VDR show that increased calcium increases expression of VDR and VDR-mediated responses.

With respect to ER studies, the first marker gene we examined was the vitamin D receptor. We have found that SERMs such as idoxifene increased VDR expression levels, as assessed by Western blot by approximately 3-4-fold, and appear to be more efficacious than estradiol. Since the VDR is a major determinant of osteoblast function, any agent that increases the expression of the receptor is likely to enhance osteoblast function, and would be expected to help maintain healthy bone structure and function. We next proceeded to characterize the ability of estrogen to modulate the production of alkaline phosphatase by these cells. In our initial studies, cells were plated and assessment of alkaline phosphatase production was made 72 hours after hormone treatment. Over this time course, we have not found any significant alteration in alkaline phosphatase production. However, MC3T3-E1 cells are representative of an early osteoblast phenotype and plans were made to culture cells under conditions that facilitate osteoblast differentiation in the presence or absence of estradiol or our test candidate SERMs. These assays have also provided preliminary data that the SERMs modestly increase cell number in comparison to untreated cultures. Studies have also been initiated to examine the effects of various SERMs on mRNA expression patterns for osteocalcin, collagen type Iα1, TGFβ and osf2. Initial results indicate that neither osteocalcin mRNA nor TGFβ mRNAs can be detected in undifferentiated MC3T3-E1 cells. Collectively, these assays will enable us to select a SERM

with good ER agonist activity (assessed by reporter gene assays) that is also able to stimulate osteoblastic function (assessed in our cellular assays) for use in animal studies (see below).

As a complement to studies performed under our standard cell culture conditions (monolayer at 1g), we have done experiments in four SLTV bioreactors. These bioreactors have enabled us to assess the effect of simulated microgravity on the ability of cells to respond to our proposed hormonal countermeasures. It was our goal to assess whether cells will respond to hormonal stimuli in this environment, and if so, to determine whether responses to our selective receptor agonists will be as effective under these conditions as they are under standard conditions. These bioreactors rotate about their horizontal axis, and in doing so, cells move only minimally relative to media while the motion of the vessel counterbalances the uniform gravitational forces cells are normally subjected to. Cells experience a net g force less than 1, are subject to low shear stresses, and can grow in three dimensions – all conditions achieved in true microgravity.

Because the cells grown in the STLVs are bound to Cytodex-1 beads, we have established techniques to prepare unit gravity cultures of MG-63 cells bound to Cytodex-1 beads to be used as controls. We found that simply mixing the cells and beads followed by plating resulted in the majority of the cells adhering to the plates. To circumvent this problem, the cells are mixed with beads and plated. One day later, the cells attached to beads are gently harvested and transferred to a fresh plate. Under these conditions, the majority of the cells at the end of the experiment, are bound to beads similar to the cells in the STLV. These cells have been used as controls for the STLV experiments described below.

In order to compare the simulated microgravity conditions of the STLV with the conditions in true microgravity, 25 million MG-63 cells were mixed with Cytodex-1 beads and grown in a 55 ml STLV rotating at 8 rpm in DMEM/F-12 + 2% charcoal stripped serum with or without calcitriol and TGFβ. Although long term culture under these conditions allows growth of multicellular aggregates containing multiple beads that might be affected by shear forces, the short-term culture of a week yields predominantly single bead suspensions with cells directly attached to the beads. We observed a reduction in alkaline phosphatase activity and collagen Iα1 mRNA compared to our Cytodex-1 bead-bound unit gravity controls. These results were consistent with the changes reported by Carmeliet *et al.* [J. Bone Miner. Res. (1997) 12:786-794] for MG-63 cells flown on the Foton 10 satellite. In subsequent studies, MEM containing 10% charcoal stripped serum with or without VDR agonists was used in order to measure osteocalcin secretion as well as our other markers. Although treatment with 100 nM calcitriol stimulated production of alkaline phosphatase activity, osteocalcin secretion, VDR expression and collagen Iα1 mRNA expression, basal levels were lower than unit gravity and stimulated levels ranged from 20-90% of unit gravity levels. The levels of calcitriol required to induce these changes cannot be attained *in vivo* because of side effects including hypercalciuria and hypercalcemia. As proposed, we have also tested the less calcemic analog, EB1089. This analog was both more potent and more effective in inducing the VDR targets; 10^{-9} M EB1089 was nearly as effective as 10^{-7} M calcitriol. Concentrations near this level should be achievable *in vivo* without eliciting hypercalciuria and hypercalcemia.

4Eii. Aim 2

In year 1, we showed that the mouse monocytic cell line, J774, as well as peripheral blood monocytes, a subset of which are thought to be osteoclast precursors, express the CaR. As an additional approach to studying the role of the CaR in osteoclastogenesis, we have started a collaboration with Dr. David Dempster in which the addition of ODF (osteoclast differentiation factor) to peripheral blood monocytes results in the formation of mature osteoclasts within 7-14 days. Thus in this more purified cellular system, it should be easier to assess the CaR's role in osteoclastogenesis, as the only cell type present is the monocyte, and it should be possible to determine the CaR's role of the formation of osteoclasts from these precursor cells. In the studies to date, we have shown that both before and after the fusion of osteoclast precursors, CaR protein expression is robust as assessed by immunocytochemistry. Additional planned studies include the use of RT-PCR, Western blot analysis and perhaps *in situ* hybridization to establish definitively at which stages of osteoclast formation the CaR is expressed and what its role is in the process of osteoclastogenesis.

4Eiii. Aim 3

We are in the process of using immunocytochemistry with anti-CaR antisera to determine which cells express the CaR in sections of normal rat and human bone as well as in bones from wild type mice and those heterozygous and homozygous for knockout of the CaR. The results to date suggest that at least some osteoclast-like cells present in bone in vivo express the CaR and could potentially mediate the known inhibitory actions of Ca^{2+}_o on osteoclast function that could make CaR agonists of possible therapeutic utility, alone or in combination with agonists of the VDR and ER, in preventing microgravity-induced bone loss. Similar studies should establish whether bona fide osteoblasts express the CaR in situ in bone

4F. Summary of Year 3 Research

4Fi. Year 3 - Aim 1

The CaR's presence in various osteoblastic cell lines indicates that this receptor is a good candidate for mediating known actions of Ca^{2+}_o to stimulate some aspects of osteoblast function. These effects of calcium would be expected to enhance bone formation by increasing the proliferation of pre-osteoblasts and inducing their chemotaxis to sites of recent bone resorption. We have not yet directly proven, however, that the actions of Ca^{2+}_o on osteoblastic cells and stromal cells are actually CaR-mediated. During year 3 we have shown in both MG-63 human and MC3T3-E1 murine osteoblastic cells that the "calcimimetic", allosteric CaR activator, NPS R-467, but not its less active stereo-isomer, NPS S-467, activate an outward K^+ channel. Because the stereoselective actions of NPS R-467 are thought to be specific for the CaR, these results provide strong support that, at least for this outward K^+ channel, its activation is CaR-mediated. In preliminary studies, we have also shown, in collaboration with Dr. Martin Pollak in the Renal Division at the Brigham and Women's Hospital, that the chemotaxis of primary mouse calvarial osteoblasts in response to elevated levels of Ca^{2+}_o is abolished in similar osteoblasts prepared from mice that are homozygous for targeted disruption of the CaR gene. Thus, if this result in

conformed in additional experiments, this chemotactic response is likewise CaR-mediated. Coupled with the data of others (Chang, et al., Endocrinology 1999; 140:5883-5893) that the CaR is expressed in most osteoblasts in sections of bovine, murine and rat bone, these data provide additional evidence proving unequivocally that the CaR is expressed in and mediates biological responses in osteoblastic cells and *bona fide* osteoblasts—an essential first step for developing CaR-based therapies--alone or in combination with VDR and ER agonists--for microgravity-induced bone loss.

As proposed, we have now completed the comparison of the actions of calcitriol with the non-secosteroidal VDR agonist, LG compound from Ligand Pharmaceuticals as well as the previously completed comparisons of the calcitriol analog EB1089 from Leo Pharmaceutical products. EB1089 is more potent and more effective than calcitriol in inducing either artificial target genes or endogenous targets including osteocalcin and alkaline phosphatase in MG-63 cells (**Figure 1**). In contrast, the non-secosteroidal agonist which is much less calcemic than calcitriol, induced a transfected target gene reasonably well, but was a poor inducer of osteocalcin and alkaline phosphatase (**Figure 2**). Consequently, the studies in rats and with rat bone marrow cells in the subsequent aims were restricted to a comparison between the most promising synthetic agonist, EB1089, and calcitriol.

Our proposed experiments using slow turning lateral vessels (STLVs) to induce a vector averaged gravity environment to simulate the effects of microgravity on MG-63 bone cells have been completed; a manuscript describing these results has been submitted (see appendix) and the results are briefly summarized here. After establishing optimal rotation conditions (8 rpm) to grow cells on Cytodex-1 microcarrier beads we compared the responses of cells to calcitriol and TGFβ grown in either the STLV or on microcarrier beads and found reduced stimulation of alkaline phosphatase expression and lower induction of Iα1 collagen mRNA in the STLV similar to the results obtained with MG-63 cells flown on the Foton 10 satellite (see attached manuscript in appendix). To test whether EB1089 could compensate for the blunted response, we switched to Minimal Essential Medium (MEM) containing charcoal-stripped serum and eliminated the TGFβ so that we could measure serum osteocalcin. The studies showed that induced levels of collagen Iα1 mRNA, alkaline phosphatase activity, and secreted osteocalcin were all reduced in the STLV in comparison to cells grown at unit gravity on microcarrier beads. EB1089 was again more potent than calcitriol and, in some cases only the EB1089 treatment differed significantly from control. An analysis of the VDR levels in these samples showed that the basal level of VDR was reduced in the STLV and the characteristic induction by calcitriol was lost; in contrast, EB1089 enhanced expression The activation states of the mitogen activated protein kinase (MAPK) and stress activated protein kinase (SAPK) signaling pathways were determined by western blotting using activation specific antibodies. Whereas there were no reproducible changes in MAPK activity, growth in the STLV activated the SAPK pathway. Treatment with calcitriol or EB1089 reduced this activation. Effects on SAPK in control cells were minimal and not significant. Thus activation of VDR can counteract some of the effects of the STLV environment. Whether activation of the SAPK pathway is a general feature of skeletal unloading remains to be determined.

VDR and its coregulators are phosphoproteins and thus their activity will be modulated by cell signaling pathways . However, little is known about the effects of altered cell signaling on VDR action. Since alterations in cytokines and in growth factors will alter intracellular signaling, we have begun to evaluate the consequences of alterations in signal transduction on

VDR activity. We have made the novel finding that inhibition of the MAPK pathway alters VDR activity in a cell-specific manner. In MG-63 cells treatment with U0126, a MEK inhibitor that blocks MAPK/Erk activation, reduces the calcitriol dependent activity both of a transfected synthetic reporter as well as secretion of endogenously produced osteocalcin (**Figure 3**) despite slightly increasing the overall levels of VDR. In contrast, treatment with U0126 enhances the VDR activity in MC3T3-E1 cells as well as facilitating calcitriol-dependent induction of alkaline phosphatase (**Figure 4**). Moreover, cotransfection of constitutively active Raf, an activator of MAPK, inhibits VDR-dependent transactivation of a transfected reporter (**Figure 5**). Inhibition of a second signaling pathway, the p38 MAPK stress activated pathway reduces activity of VDR in both cell types (**Figure 6**). Thus, we find that altered cell signaling, likely to be encountered in organisms in a microgravity environment, has a profound effect on the activity of the VDR. A manuscript reporting these results is in preparation.

In addition to the studies utilizing cell lines, in the third year we have initiated studies utilizing rats. In pilot studies done in collaboration with Dr. Dana Gaddy-Kurten (University of Arkansas), we looked at the effect of hindlimb elevation on bone cell precursors in 6 month old male rats. After one month of suspension, bone marrow was harvested from suspended animals and the ability of bone marrow cells to differentiate into osteoblasts was compared to control animals. Suspension reduced the number of osteoblast precursor cells measured as alkaline phosphatase positive colonies (**Figure 7**) 10 days after plating compared to control. Both EB1089 and calcitriol corrected this deficiency. More detailed studies shown in progress for aim 2, demonstrated that EB1089 is more potent in inducing osteoblast formation.

Our previous studies of SERMs in MG-63 cells and MC3T3-E1 cells demonstrated that 17β-estradiol had clear agonist activity in transient transfection assays in which synthetic target genes were introduced into cells. However, when various SERMs were tested in the same model, the activity exerted by these compounds was significantly less than estradiol. Since several of these compounds (e.g. tamoxifen) have been shown *in vivo* to be effective replacements for estrogen deprivation, we sought to develop a more sophisticated model in which to assess their effect on osteoblast differentiation and/or function. Therefore, we performed studies in MC3T3-E1 cells that had been differentiated over a 28-day period in 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. These studies were performed with estradiol, idoxifene (the SERM that had exhibited the most agonist activity in our previous assays) and raloxifene (a SERM that has FDA approval for osteoporosis applications in humans).

In our first study, MC3T3-E1 cultures were treated with ascorbic acid and β-glycerophosphate in the presence of vehicle, 1 nM 17beta-estradiol, 100 nM raloxifene or 100 nM idoxifene for up to 28 days. Cells were harvested at 7, 14, 21 and 28 days for measurement of alkaline phosphatase activities, and found to be elevated 2 to 4-fold in estradiol-treated cells (**Figure 8**). Values obtained for cells cultured in the presence of raloxifene or idoxifene were not different than values obtained for vehicle-treated cultures. Cells cultured in parallel were harvested for RNA isolation and subsequent measurement of various target genes. Northern blot analysis was used to measure levels of collagen-Iα1 mRNA which were increased over the time course of the differentiation in the absence of hormone treatment (**Figure 9**), indicating that these cells were progressing down an osteoblastic differentiation pathway. Treatment of cells with estradiol for 14, 21 or 28 days reduced collagen mRNA levels by up to 50% in comparison to vehicle-treated cultures (**Figure 10**) In contrast, idoxifene significantly increased, while raloxifene had little effect, on type one collagen mRNA levels at 21 and 28 days. Similar patterns

were found in hormone-induced alterations in osteonectin mRNA expression (**Figure 11** However, the expression profiles of estradiol relative to SERMs were not similar for every target gene examined. For example, at 21 days both estradiol and idoxifene increased steady-state TGFβ mRNA levels while expression in raloxifene-treated cells was not significantly different than in ethanol-treated control cultures (**Figure 12).** At 28 days, hormone-induced changes in TGFβ mRNA expression were markedly attenuated. No changes in osteocalcin mRNA expression were detected after 21 or 28 days of culture (**Figure 13**). Collectively, these data suggest that estradiol and SERMs mediate distinct effects on the ability of differentiating MC3T3-E1 cells to express markers of osteoblast function. Furthermore, these data indicate that idoxifene and raloxifene are not biologically equivalent and that it is possible to distinguish between SERMs with respect to activation of gene expression in osteoblastic cells. Taken together with our reporter gene assays, these results raise the possibility that estradiol and SERMs exert their protective effects in the skeleton through distinct and/or novel pathways. It is striking that the agents studied in this experiment are all effective osteoprotective agents *in vivo*, even though their relative abilities to activate synthetic gene expression (e.g. in transient transfection assays) and endogenous gene expression (e.g. collagen mRNA expression levels) are unique, suggesting the possibility that the estrogen receptor-mediated events in bone important for maintaining this tissue's structure and function may be distinct from responses important for reproductive function.

4Fii. Aim 2

The CaR's presence in osteoclast precursors and in osteoclast-like cells formed *in vitro* supports the receptor's potential role in regulating osteoclastogenesis and potentially mediating the known inhibitory actions of high Ca^{2+}_o on the function of mature osteoclasts. During year 3, we have obtained additional evidence for the CaR's presence and/or its functional relevance in putative osteoclast precursors and/or mature osteoclasts using cultured cell models. In collaboration with Dr. David Scadden's laboratory at the Massachusetts General Hospital, we have shown that the CaR mediates the chemotactic actions of high Ca^{2+}_o on peripheral blood monocytes, utilizing a combination of pharmacological (e.g. the use of NPS R-467 and S-467) and genetic tools (the use of monocytes from CaR knock-out mice). The CaR acts synergistically with other peptide chemokines for monocytes [e.g. macrophage chemotactic peptide-1 (MCP-1)] in this regard, doing so, in part, by upregulating its own receptor as well as those for the chemokines. Thus monocytes may utilize the CaR to move to sites of recent bone resorption. Since these cells are known to release substances in response to high Ca^{2+}_o that tend to promote osteoblastic activity and inhibit osteoclastic activity, the CaR on monocytes could serve as a target for potentially beneficial actions of a CaR activator on the balance between bone resorption and formation. We have also characterized further the model of osteoclastogenesis utilized by Dr. Dempster in collaborative studies (see description above for studies in Year 2) and utilized RT-PCR as well as Western blotting with a CaR-specific antibody to show that both precursors of osteoclasts as well as mature multinucleated osteoclasts formed in this culture system from peripheral blood monocytes express CaR transcripts and protein. NPS R-467 and S-467, however, only inhibited osteoclast function at very high levels and with similar potencies, suggesting that the CaR does not couple to the regulation of bone resorption in this *in vitro* model of osteoclast biology. Further studies are needed to determine whether the receptor regulates other biologically relevant functions in this experimental system, including

osteoclastogenesis, cytokine production, chemotaxis of OC precursors, etc., that would be relevant to the possible development of CaR-based therapies—alone or in combination with ER and VDR agonists—that reduce bone resorption in microgravity-induced bone loss.

For our VDR studies, rat bone marrow cultures have been utilized as a model to study osteoclast differentiation. To compare the response of bone marrow cells to calcitriol and EB1089, bone marrow was harvested, plated in medium to induce osteoblast or osteoclast differentiation and treated with varying levels of calcitriol or EB1089. In our initial studies, EB1089 was more potent and more efficacious in inducing osteoblast precursors judged by ALP staining, but was less active in inducing differentiation to multinucleated osteoclasts (**Figure 14**). Similar results were obtained by Dr. Gaddy-Kurten utilizing bone marrow from hindlimb suspended animals. A major role of calcitriol in stimulating osteoclast differentiation is its induction in stromal cells of RANKL that binds to RANK on the osteoclast precursors stimulating differentiation of the osteoclasts. To determine whether calcitriol and EB1089 differ in their ability to induce RANKL, bone marrow cells from hindlimb suspended animals were cultured under conditions optimized to induce osteoclast formation in medium containing vehicle, calcitriol, or EB1089. Our initial studies indicate that calcitriol induces RANKL as expected, but that EB1089 does not. This study is shown in progress for aim 3 describing studies utilizing *in vivo* treatments.

4Fiii. Aim 3

Our studies to date strongly suggest that the CaR is expressed in *bona fide* bone cells as well as in various osteoblastic bone cell lines and osteoclasts formed *in vitro*. In addition the studies of Chang, *et al.* in sections of murine, rat and bovine bone described above strongly supported the CaR's expression in osteoblasts *in vivo*, although further studies are needed to assess the range of functions that it regulates and their relevance to osteoblast physiology and pathophysiology.

Such studies will be aided by the development of mice with knockout of the CaR that have been "rescued" from the severe hypercalcemia and hyperparathyroidism that have limited to date the utility of this model for studying the role of the CaR *in vivo*. Such a rescue now appears to be feasible by crossing mice with CaR knockout with those that have recently been developed in which the PTH gene has been knocked out, thereby preventing hyperparathyroidism despite the lack of the CaR in parathyroid gland. This model system should also facilitate studies on the CaR's relevance to osteoclast biology *in vivo*, as there is still uncertainty regarding its functional relevance to known inhibitory actions of high Ca^{2+}_o on osteoclastogenesis and the resorptive activity of mature OCs. Once the CaR's role in these two processes as well as in other osteoclastic functions have been established—for example, in studies utilizing osteoclasts formed *in vitro* from CaR knockout monocytes using Dr. Dempster's culture system—it should be possible to obtain a clearer sense of the CaR's potential as a target for anti-resorptive therapy in microgravity-induced bone loss.

As a complement to our ongoing assessment of both VDR and ER agonists in our cellular models we have begun to assess the effect of various VDR and ER agonists in rats. With respect to our ER/SERMs studies, we had originally proposed to examine the effects of SERMs on male and female reproduction. This was an important consideration because of the possibility that these drugs may impair normal reproductive function outside of the skeletal system. Based on

our data obtained in years one and two, we had anticipated that idoxifene would be the lead compound for testing in our rat studies. However, recent human clinical trial results of idoxifene indicate that female human exposure to this compound leads to uterine hyperplasia of an unacceptable magnitude (Larry Suva, SmithKline Beecham, personal communication). In view of this, we decided not to test idoxifene in our rat studies because at the present time it seems unlikely that idoxifene will receive FDA approval for human use, and these studies would not further NSBRI objectives of advancing human exploration of Space. Therefore, we have pursued our rat studies with raloxifene, a SERM that has received FDA approval for osteoporosis applications. Previous animal studies have demonstrated that raloxifene prevents ovariectomized-induced bone loss in female rats and established an appropriate dose range, administered by oral gavage, that will maintain bone density in an environment of estrogen deprivation. Assessment of raloxifene on reproduction and overall safety has been completed by the manufacturer of this compound [[see for example, Reproductive Toxicology 12:217-221, 1998; Reproductive Toxicology 12:223-232, 1998]]. We therefore proceeded to experiments that have enabled us to work towards testing the effect of raloxifene, in a hindlimb-suspended rat model.

We have addressed several questions in year 3 with respect to SERMs usage in mature, six-month old female rats. The first relates to the method of SERM administration. Published doses administered via oral gavage have been determined that are able to counteract the loss of estrogen associated with ovariectomy with respect to bone mineral density and biochemical markers of bone metabolism. However, this delivery method is not suitable for hindlimb unloaded animals because of the amount of daily handling required greatly increases the risk the hindlimbs will become weight bearing, and that rats will damage their tails and/or the device that provides the hindlimb suspension. Therefore, we needed to administer agents by an alternative method for our studies. Time release pellets were chosen because they can be implanted prior to hindlimb suspension, will deliver a uniform amount of drug over the 28 day hindlimb suspension period, and have been used previously by us and other investigators to deliver steroid receptor ligands to rats and mice. Our first goal was to ensure that the raloxifene dosage chosen for our studies was sufficient to prevent bone loss induced by ovariectomy. Based on a) the relative amounts of raloxifene required to maintain bone mineral density in comparison to estradiol by oral gavage; b) the amounts of estradiol and tamoxifen that have been administered by time release pellet and exerted a biological effect in previous studies, and c) consultation with the manufacturer of the time release pellets (Innovative Research of America), we had time release pellets manufactured that would release 535 μg raloxifene per day. As a positive control, we also obtained pellets that would release 12 μg 17β-estradiol per day as well as placebo pellets. In addition, since at least one study has been performed in which the SERM tamoxifen was administered via time release pellet and able to counteract ovariectomy-induced reduction in bone mineral density (Bone 17: 181S-190S, 1995), we also included tamoxifen (583 μg/day) in this experiment. Six-month old, virgin Sprague Dawley rats were ovariectomized and implanted with either placebo, 17β-estradiol, raloxifene or tamoxifen pellets. In addition, another control group of sham operated animals was included in this study. Animals were then housed for 28 days at which time they were euthanized and samples collected for analysis to assess the relative ability of each treatment to affect bone mineral density and other biochemical markers.

As an independent biological marker of the test compounds, we monitored uterine weights. As anticipated, ovariectomy resulted in a 40% decrease in uterine weight in comparison to the

sham-operated group. Estradiol increased uterine weight relative to placebo, while raloxifene and tamoxifen were lower indicating that the estradiol dosage was sufficient to exert a classical, estrogen-dependent, uterotrophic response, while the SERM dosages lack any estrogenic activity in the uterus (**Figure 15**). Serum osteocalcin levels measured by RIA revealed that ovariectomy increases osteocalcin values by 35% relative to sham operated animals indicating an increase in bone turnover. Estradiol treatment reduced osteocalcin values by 60%, while tamoxifen and raloxifene reduced osteocalcin by 30% and 20% respectively demonstrating that all agents could reduce the extent of bone turnover (**Figure 16**). In addition, pyridinium crosslinks, normalized to creatinine, were reduced in estradiol, raloxifene and tamoxifen treated rats in comparison to placebo controls, consistent with the ability of these ER ligands to reduce bone turnover (**Figure 17**). Femurs were removed from these animals at sacrifice, and the distal segment (measured 5, 5.5 and 6 mm from the most distal aspect) was subjected to analysis by pQCT (in collaboration with Dr. Susan Bloomfield, Texas A&M University) enabling us to determine total bone content, total bone density, cortical bone density, trabecular bone density, total area, trabecular area and cortical area. In comparison to placebo values, significant differences were noted in total bone content (E2 and raloxifene), total density (E2, raloxifene and tamoxifen), trabecular density (E2, raloxifene and tamoxifen), trabecular area (E2 and tamoxifen) and cortical area (E2 and tamoxifen) (see for example **Figure 18**). Measurements taken at the mid-shaft region were significantly different from placebo values for cortical content (E2 only). Collectively, these data indicate the dosage of raloxifene chosen for these and the following studies is sufficient to prevent bone density loss in ovariectomized animals, and are therefore appropriate for further evaluation (see below).

In our second SERM experiment, we assessed whether SERM administration to ovary intact females would impact skeletal biology, in order to ensure that SERM administration would not compete with endogenous estrogens and potentially have a negative consequence on bone metabolism. Therefore, intact, six-month old, virgin Sprague Dawley rats were implanted with either placebo, estradiol, raloxifene or tamoxifen pellets (same dosage as above) and animals were housed for 28 days after which time, uteri, sera, urine and bones were collected for analyses. Both raloxifene and tamoxifen antagonized endogenous estrogen action as evidenced by decreased uterine weights, collectively indicating that hormones were delivered to these animals (**Figure 19**). Although the effects were not significant, tamoxifen and raloxifene reduced levels of serum osteocalcin (~40%) and urinary pyridinium crosslinks (~10-20%). In contrast, estradiol treatment had no effect on osteocalcin levels but did significantly lower deoxypyridinoline by ~40% (**Figures 20 and 21**). Femurs were removed from these animals at sacrifice, and the distal segment (measured 5, 5.5 and 6 mm from the most distal aspect) and mid-shaft region were subjected to analysis by pQCT enabling us to determine total bone content, total bone density, cortical bone density, trabecular bone density, total area, trabecular area and cortical area. No significant changes were noted (see for example **Figure 22**). Thus, administration of the SERM, raloxifene, does not negatively impact bone metabolism in normal, adult females, and should not therefore increase the risk of bone loss in hindlimb suspended animals.

We recognize that testing our candidate countermeasures in an animal model of disuse is of extreme importance with respect to evaluating countermeasures for microgravity-induced bone loss. Therefore, we submitted a supplementary proposal outlining experiments to test our ER-based (and VDR-based, see below) countermeasures in hindlimb suspended animals in

collaboration with Dr. Susan Bloomfield at Texas A&M University. Based on funding, this study was initiated in March, 2000 and has funding through til December 2000. The design of the ER component of this study is to ovariectomize 5 month-old, virgin Sprague Dawley female rats and allow the animals to recover over a 28 day period. At that point, animals were divided into three groups and received either placebo, estradiol or raloxifene time release pellets and were subjected to hindlimb suspension for 28 days. This study was performed with ovariectomized females to ensure that our assessment of hormone treatments reflected the ability of raloxifene and estradiol to counteract unloading-induced bone loss, not their ability to substitute for endogenous estrogens that may be reduced as a result of the changing endocrine environment that occurs as a result of hindlimb suspension. While data are not yet available for female rats, it is clear that male rats undergo a dramatic and sustained change in their levels of circulating sex steroids as a result of hindlimb suspension and Spaceflight. At this point, we have partially completed these studies and preliminary, incomplete pQCT data is reported on the animals examined to date (n=3 for placebo, n=4 for estradiol, n=4 for raloxifene) at the proximal tibia (scanned 5, 5.5 and 6 mm from the proximal plateau of the right leg). The remainder of the animals (n=10 per group) will be completed in the next three months and biochemical assays (e.g. osteocalcin, pyridinium crosslinks, creatinine) will be performed when all animal manipulations are complete. We have found that estradiol and raloxifene treatment maintains bone mineral density during the hindlimb suspension period (**Figure 23**). Interestingly, the data collected to date suggests that this overall effect results from an estradiol or raloxifene-induced increase in cortical bone density that compensates for the loss in trabecular bone mineral density. These results point to potential differences in the mechanisms by which ER- and VDR-based countermeasures may achieve their effects (e.g. ER agonists have a stronger effect on the cortical compartment, while VDR-based countermeasures appear to exert a greater effect on the trabecular compartment – see below) and supports our hypothesis that combinations of ER and VDR receptor ligands may represent a significantly more effective therapy than either agent alone.

As a preliminary experiment to treating hindlimb elevated rats with EB1089, we have established doses to deliver EB1089 through Alzet pumps that do not induce either hypercalciuria or hypercalcemia after a 28 day treatment. We find that neither 0.25 μg/kg/day nor 0.05 μg/kg/day induces hypercalcemia in 6 month old male rats (**Figure 24**), but the higher dose causes mild elevation in urine calcium. Induction of kidney 24 hydroxylase, a marker of calcitriol action, was higher in the high dose animals. Based on these studies, a dose of 0.1 μg/kg/day is being used for the hind limb elevation experiments that are being done under the supplement that was begun in March. These studies will continue through the end of the year, but the initial results are summarized below.

All hindlimb elevation studies are carried out at Texas A&M under the supervision of Dr. Susan Bloomfield. Two sets of four animals each staggered by 1 day are treated simultaneously. Ultimately, 10 animals will receive each treatment. Primed Alzet pumps delivering vehicle, 0.1 μg/kg/day calcitriol or 0.1 μg/kg/day EB1089 were inserted subdermally and initial pQCT measurements on the proximal and mid-diaphysis tibia were made immediately prior to hindlimb elevation. After 28 days, animals were again anesthetized, and pQCT measurements made. Animals were sacrificed, and samples including femurs for bone marrow extraction, blood, urine, kidneys, and intestine were collected. Femurs were transported to Baylor within a few hours of excision, bone marrow isolated, and cells plated for differentiation to osteoblasts and osteoclasts.

Analyses on the animals completed to date reveal that 0.1 μg/kg/day of calcitriol somewhat elevates serum calcium levels as we had expected, but that there is no effect of EB1089 at this level (**Figure 25**). Analysis of urine calcium normalized to creatinine shows a substantial elevation in the calcitriol treated animals, but minimal and statistically insignificant effects in the EB1089 treated animals (**Figure 25**). Urine pyridinium crosslinks are slightly elevated in the urine of calcitriol treated animals, but not in the EB1089 treated animals (**Figure 26**). This is consistent with calcitriol enhancing the activity of the osteoclasts.

Analyses of the tibia of the control animals showed statistically significant losses in bone mineral density (BMD), trabecular bone mineral density, and cortical bone area in the proximal tibia. *In each case, EB1089 prevented these losses* (**Table I**). Interestingly, calcitriol actually increased BMD, but the elevated serum and urine calcium levels would preclude the use of this level in humans. Analyses of the cells derived from the femurs suggest that EB1089 stimulates maturation of osteoblasts. The % alkaline phosphatase positive colonies after 10 days of growth was higher in animals that had received EB1089 than in calcitriol or control animals (**Figure 27**), but there appears to be a reduction in the total colony forming units (**Figure 27**). Cells grown from control, calcitriol or EB1089 treated animals all contain similar low levels of RANKL measured by western blotting. Treatment of these cells with 10 nM calcitriol induces RANKL. *Strikingly, treatment with 10 nM EB1089 does not induce RANKL!* (**Figure 28**). This surprising difference may be responsible for the difference in osteoclast activity *in vivo*. In summary, the preliminary results suggest that EB1089 can be utilized to prevent bone loss induced by hind limb elevation in mature male rats. This is accomplished without unacceptable increases either in serum or urine calcium.

5. Implications of Project Findings for Future Research

The results of the project have several implications for future research. With respect to the CaR studies, it is clear that the CaR is expressed in bone and in bone precursor cells, and that this receptor appears to play roles in important processes such as chemotaxis which may contribute to the processes by which cells are recruited to sites of bone metabolism. Future work is warranted to examine in more detail the role of the CaR in bone biology.

The VDR studies indicate that osteoblastic gene expression is compromised under conditions which mimic several aspects of microgravity. The responsiveness of these genes to the natural VDR ligand, calcitriol, is reduced, and cell signaling pathways are altered suggesting that more research is required to better understand the cellular and molecular changes that accompany microgravity. Nevertheless, a more potent VDR agonist can partially overcome the simulated microgravity-induced reduction in gene expression in osteoblastic cells and when used in animals this compound appears able to prevent hindlimb suspension-induced bone loss. This suggests that a VDR-based countermeasure will be useful to prevent bone loss and this deserves further investigation in animal models of disuse.

The ER studies reveal that estradiol and SERMs, although both able to prevent bone loss associated with sex steroid depletion, do not appear to regulate osteoblastic gene expression in a similar manner. This suggests that estradiol and SERMs either regulate bone mineral density through different pathways or that the molecular events through which the ER regulates bone density are distinct from our current understanding of ER action. Either possibility should be further explored because it has the potential to reveal new mechanisms for enhancing bone

structure and function that may facilitate the identification of a high potency, high specificity agents for treating osteoporosis. In addition, either estradiol or raloxifene appears able to prevent hindlimb suspension-induced bone loss in preliminary studies. This deserves further investigation in animal models of disuse.

Finally, our studies have revealed that the absence of mechanical loading and/or gravitational forces results in changes that can be characterized at the molecular, cellular and organismal levels. Further study of these changes has the potential to delineate events critical to these alterations that may reveal novel approaches to the prevention or treatment of microgravity-induced bone loss.

6A. Project Research Data

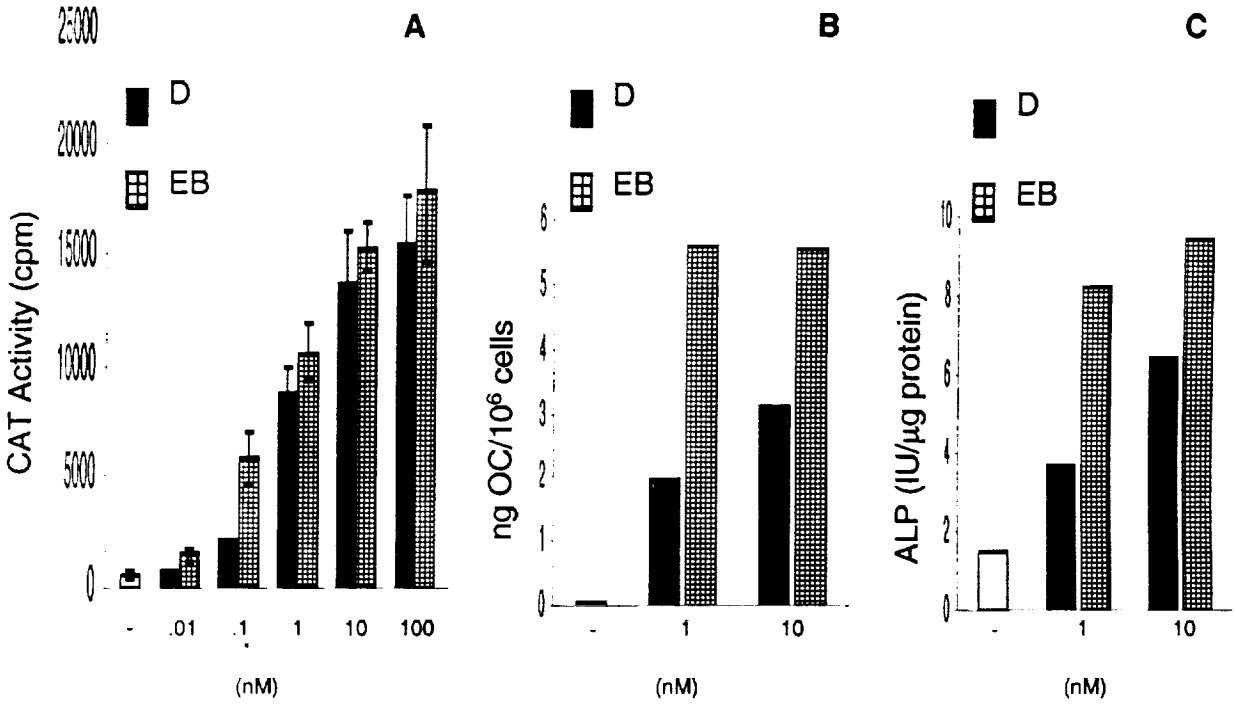


Figure 1: Vitamin D Receptor agonist, EB1089, is more potent than Calcitriol in MG-63 cells. MG-63 cells were treated with the indicated concentrations of EB1089 or calcitriol and analyzed for transactivation (A), Osteocalcin (B) and Alkaline Phosphatase (C). Open bars indicate vehicle treatment, solid bars are calcitriol treated and hatched bars are EB1089 treated cells.

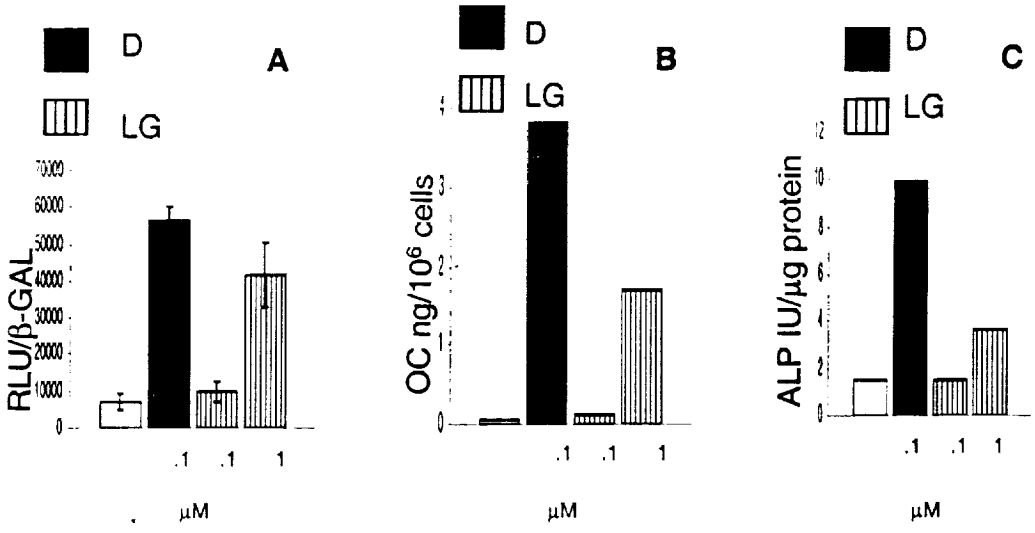


Figure 2: Calcitriol is more effective than the non secosteroidal VDR agonist, LG 190119 in the induction of transactivation, osteocalcin and Alkaline Phosphatase in MG-63 cells. MG-63 cells were transfected or not with VDRE-LUC and assayed for transactivation (A), Osteocalcin (B) and Alkaline Phosphatase (C).

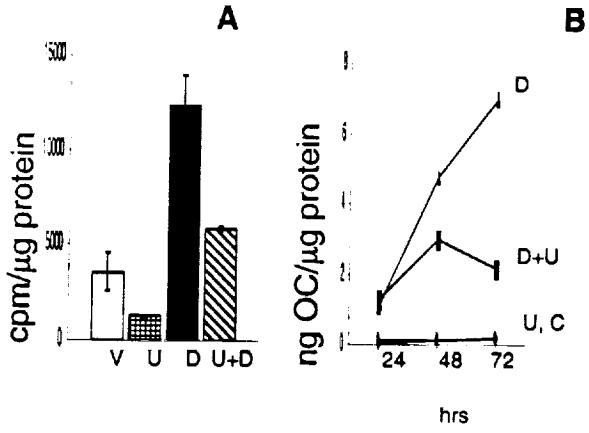


Figure 3: Effect of MEK inhibitor, UO126, on transactivation and osteocalcin secretion in MG-63 cells. A. MG-63 cells were transfected with VDRE-tk-CAT, treated with vehicle (V), 20 µM UO126 (U), 1 nM calcitriol (D) or a combination of UO126 and calcitriol and assayed for CAT activity after 24 hrs. B. MG-63 cells were treated with vehicle (C), 20 µM UO126 (U), 100 nM calcitriol (D) or a combination of UO126 and calcitriol (D+U) for the indicated times. Osteocalcin secretion into the medium was measured and normalized to the total protein content.

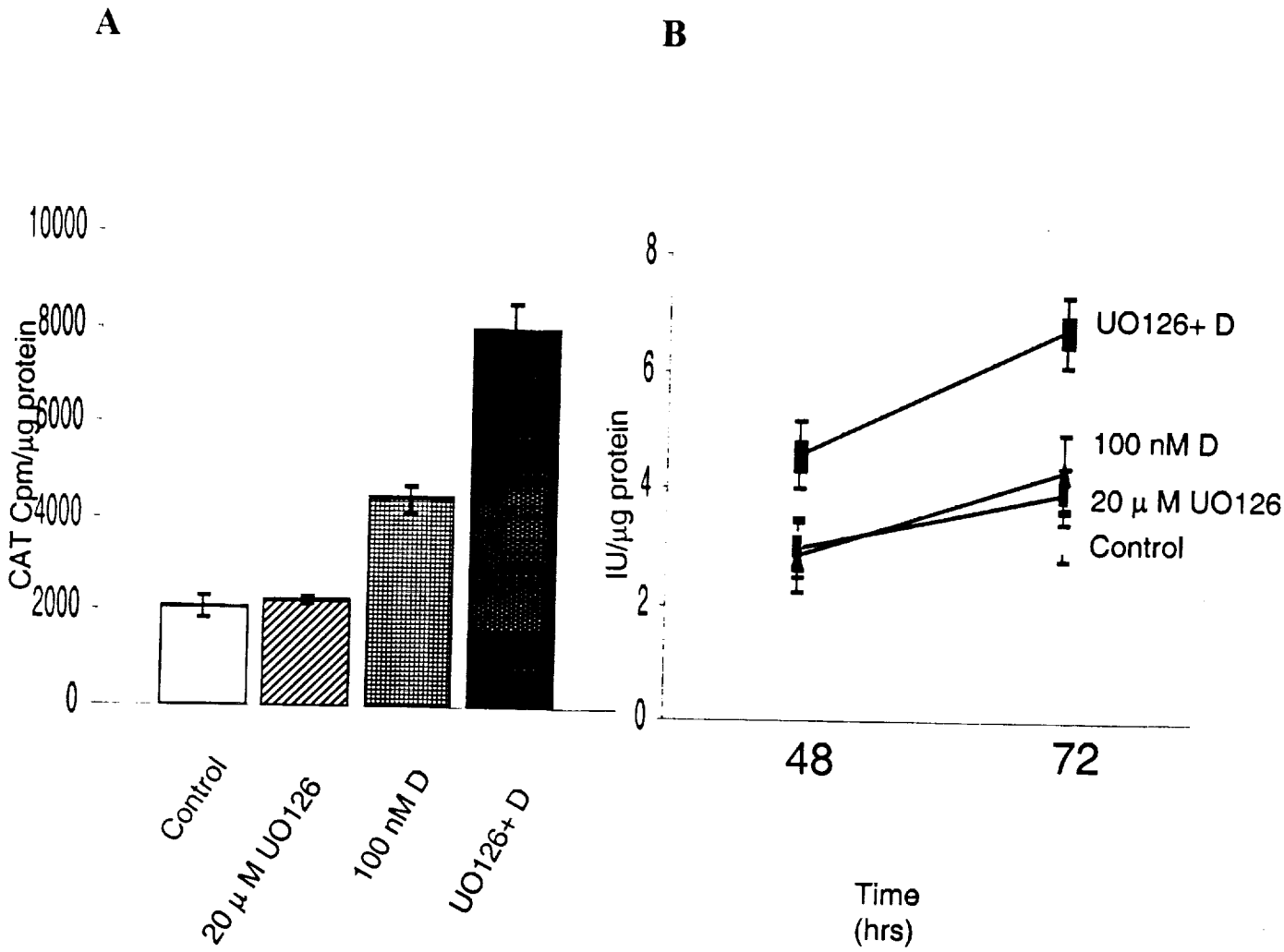


Figure 4: ERK is an inhibitor of Vitamin D mediated gene transcription in MC3T3-E1 cells. A. MC3T3-E1 cells were transfected with VDRE-tk-CAT, treated with vehicle, 20 µM UO126, 1 nM calcitriol or a combination of UO126 and calcitriol and assayed for CAT activity after 24 hrs. B. MC3T3-E1 cells were treated with vehicle, 20 µM UO126, 100 nM calcitriol or a combination of UO126 and calcitriol (D+U) for the indicated times. Total cellular alkaline phosphatase was measured and normalized to the total protein content.

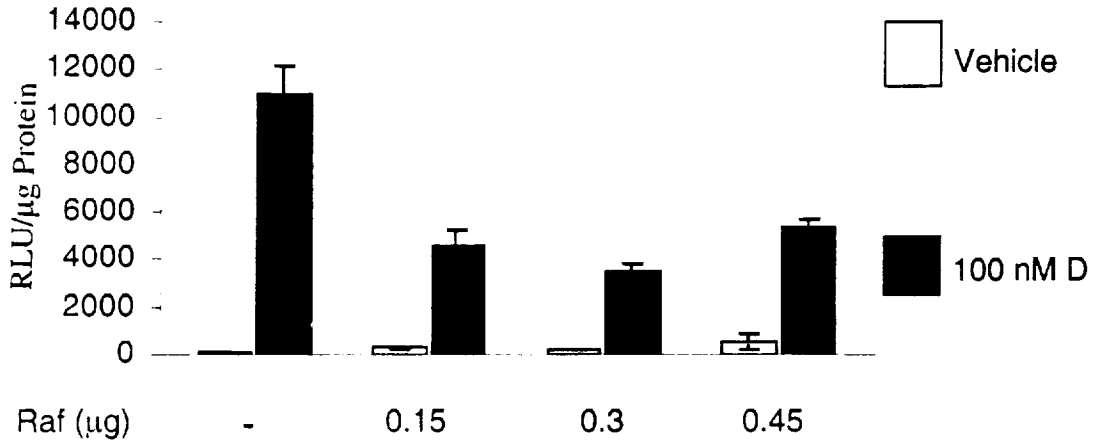


Figure 5: Cotransfection of Raf-1 inhibits the transactivation of Vitamin D Receptor in MC3T3-E1 cells. MC3T3-E1 cells were transfected with VDRE-LUC and the indicated concentrations of Raf-1 balanced with empty vector by an adenovirus mediated method and treated with vehicle or calcitriol. Open bars indicate vehicle treatment and solid bars indicate 100 nM calcitriol treatment.

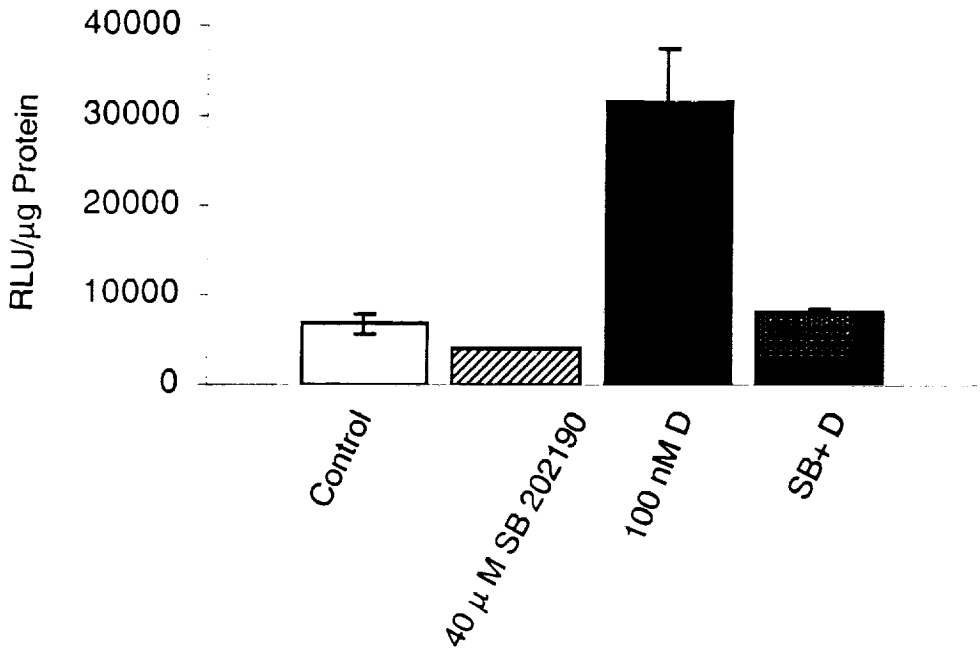


Figure 6: p38 MAPK is an activator of Vitamin D Receptor mediated gene transcription in MC3T3-E1 cells. MC3T3-E1 cells were transfected with VDRE-LUC and treated with vehicle, SB202190, calcitriol or SB202190 and calcitriol.

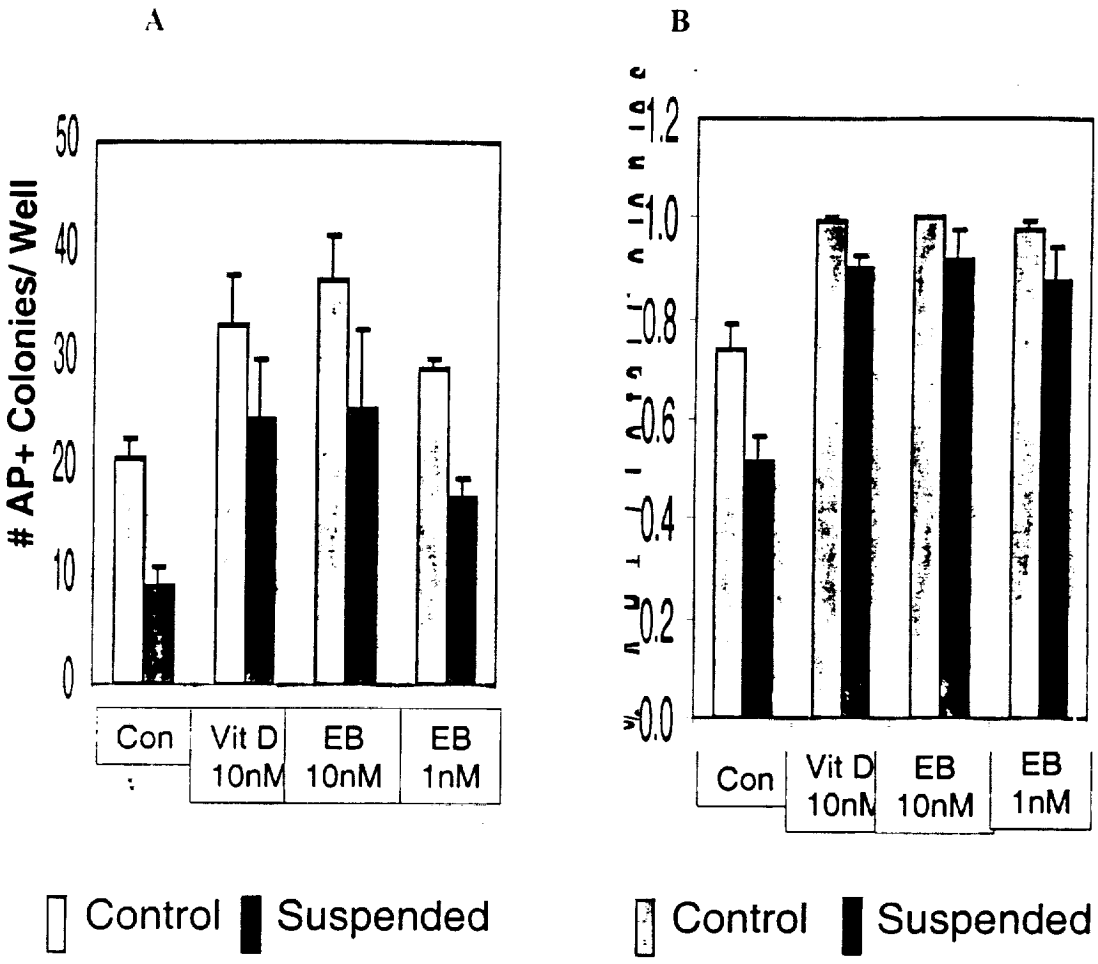


Figure 7: Calcitrol (vit D) and EB1089 (EB) increase the number of alkaline phosphatase-positive osteoblastic cells derived from bone marrow obtained from control and hindlimb suspended rats.

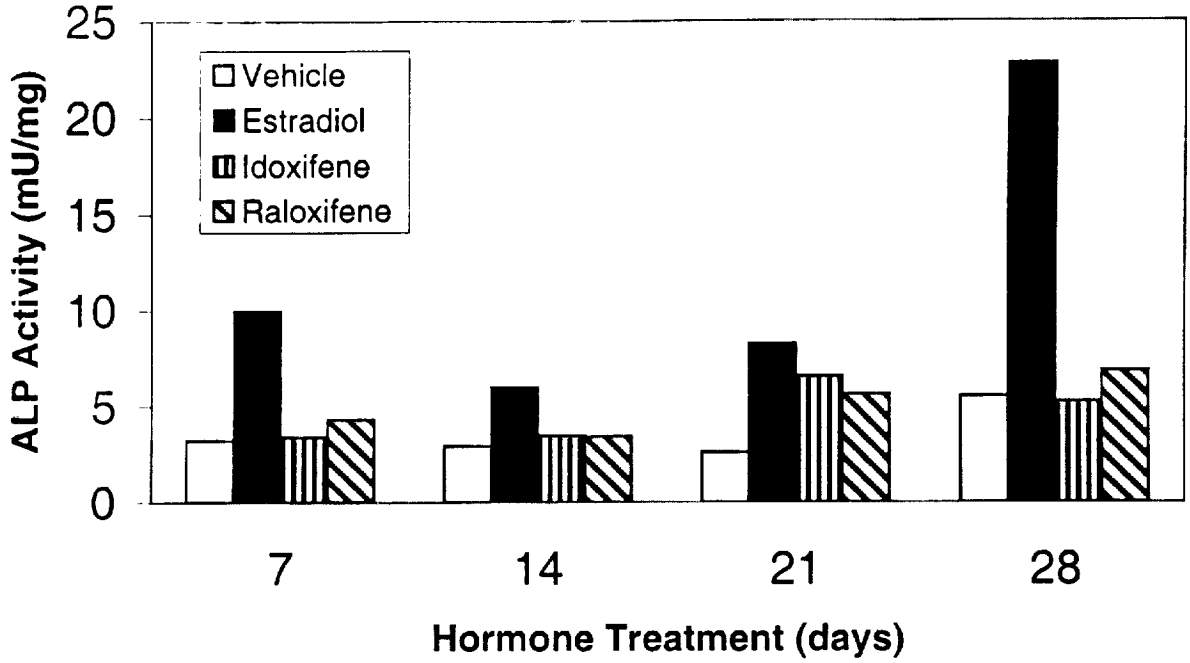


Figure 8: Regulation of alkaline phosphatase activity by ER ligands in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 10 mM βglycerophosphate and 50 μg/ml ascorbic acid for up to 28 days in the presence of 10 nM 17β-estradiol, 100 nM Idoxifene, 100 nM Raloxifene or vehicle (ethanol). Cells were harvested and alkaline phosphatase activity determined and standardized to cellular protein.

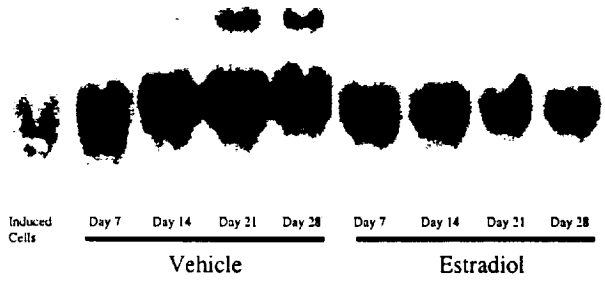


Figure 9: Expression levels of collagen type Iα1 mRNA levels and regulation by estradiol in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 10 mM βglycerophosphate and 50 μg/ml ascorbic acid for 21 or 28 days in the presence of 10 nM 17β-estradiol or vehicle (ethanol). Cells were harvested and RNA extracted for subsequent Northern blot analysis. Top panel, Northern blot analysis of collagen mRNA. Bottom panels, Northern blot analysis of cyclophilin mRNA. The graphs represent collagen mRNA levels standardized to cyclophilin.

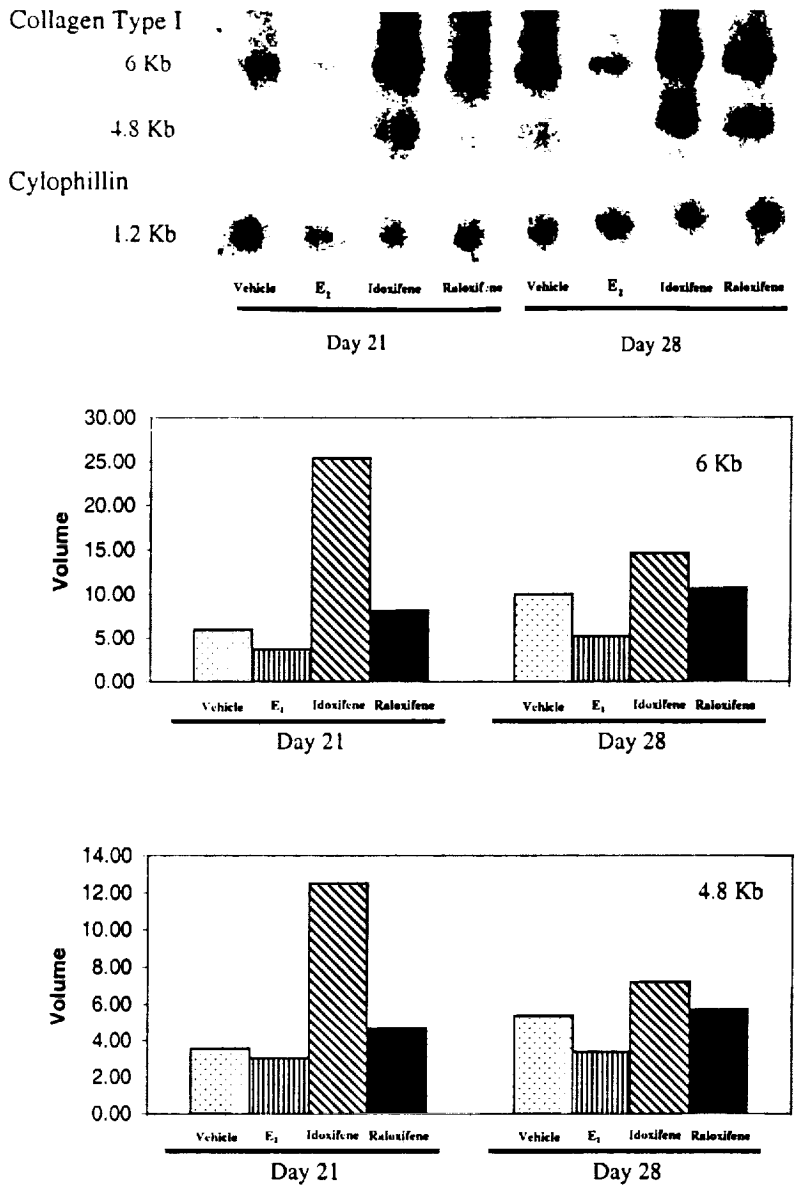


Figure 10: Regulation of collagen type Iα1 mRNA levels by ER ligands in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 10 mM βglycerophosphate and 50 μg/ml ascorbic acid for 21 or 28 days in the presence of 10 nM 17β-estradiol, 100 nM Idoxifene, 100 nM Raloxifene or vehicle (ethanol). Cells were harvested and RNA extracted for subsequent Northern blot analysis. Top panel, Northern blot analysis of collagen mRNA. Middle panel, Northern blot analysis of cyclophilin mRNA. The graphs represent collagen mRNA levels standardized to cyclophilin.

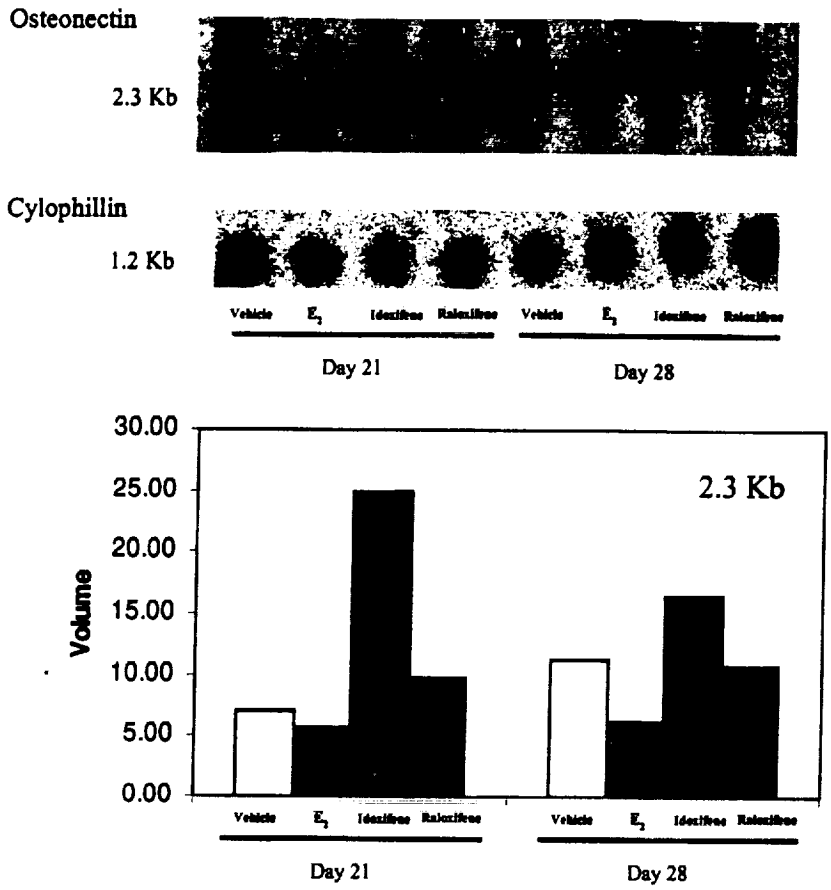


Figure 11: Regulation of osteonectin mRNA levels by ER ligands in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 10 mM βglycerophosphate and 50 μg/ml ascorbic acid for 21 or 28 days in the presence of 10 nM 17β-estradiol, 100 nM Idoxifene, 100 nM Raloxifene or vehicle (ethanol). Cells were harvested and RNA extracted for subsequent Northern blot analysis. Top panel, Northern blot analysis of osteonectin mRNA. Middle panel, Northern blot analysis of cyclophilin mRNA. The graph represents osteonectin mRNA levels standardized to cyclophilin.

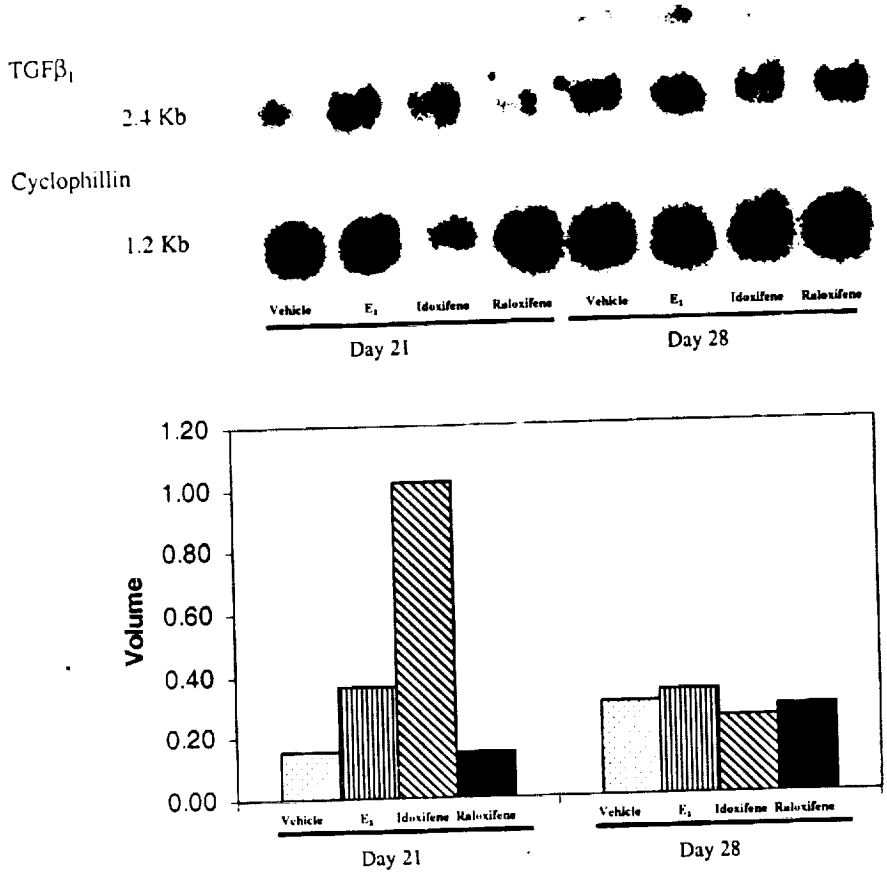


Figure 12: Regulation of TGFβ mRNA levels by ER ligands in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 10 mM βglycerophosphate and 50 μg/ml ascorbic acid for 21 or 28 days in the presence of 10 nM 17β-estradiol, 100 nM Idoxifene, 100 nM Raloxifene or vehicle (ethanol). Cells were harvested and RNA extracted for subsequent Northern blot analysis. Top panel, Northern blot analysis of TGFβ mRNA. Middle panel, Northern blot analysis of cyclophilin mRNA. The graphs represent TGFβ mRNA levels standardized to cyclophilin.

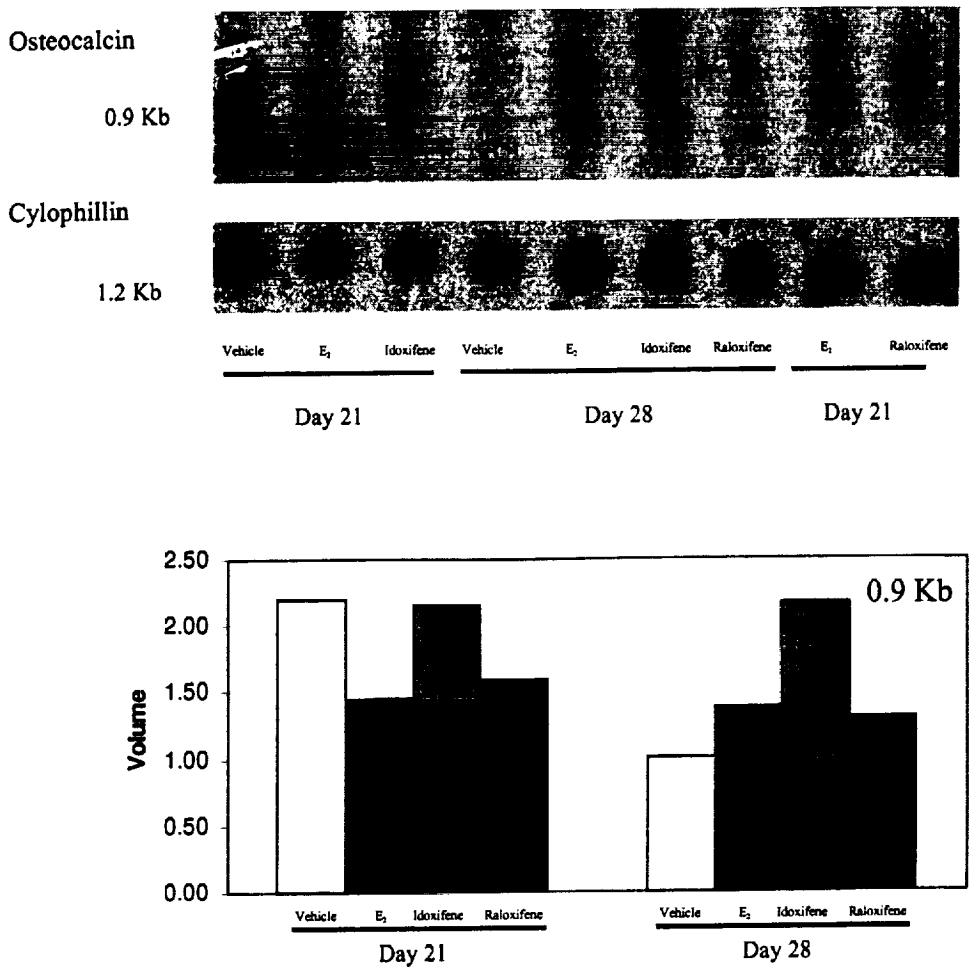


Figure 13: Regulation of osteocalcin mRNA levels by ER ligands in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence of 10 mM βglycerophosphate and 50 μg/ml ascorbic acid for 21 or 28 days in the presence of 10 nM 17β-estradiol, 100 nM Idoxifene, 100 nM Raloxifene or vehicle (ethanol). Cells were harvested and RNA extracted for subsequent Northern blot analysis. Top panel, Northern blot analysis of osteocalcin mRNA. Middle panel, Northern blot analysis of cyclophilin mRNA. The graphs represent osteocalcin mRNA levels standardized to cyclophilin.

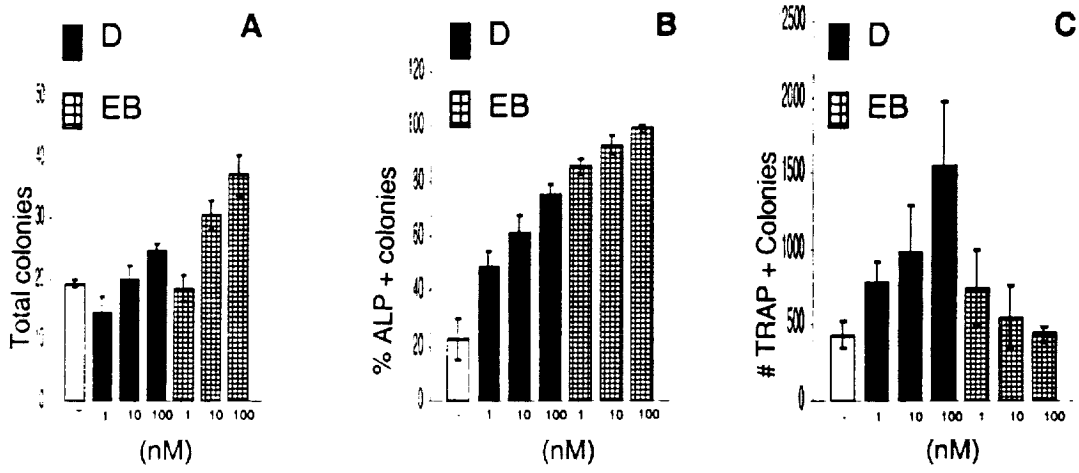


Figure 14: EB1089 activates fibroblasts and not osteoclasts in rat bone marrow primary culture. Primary bone marrow cell cultures from six month old rats were treated with vehicle (open bars) or the indicated concentrations of calcitriol (solid bars) or EB1089 (hatched bars). At the end of 10 days of culture, the total colony forming unit fibroblasts (A), percent colonies stained positive for Alkaline Phosphatase (B) or Total Tartrate Resistant Acid Phosphatase staining colonies (C) were determined.

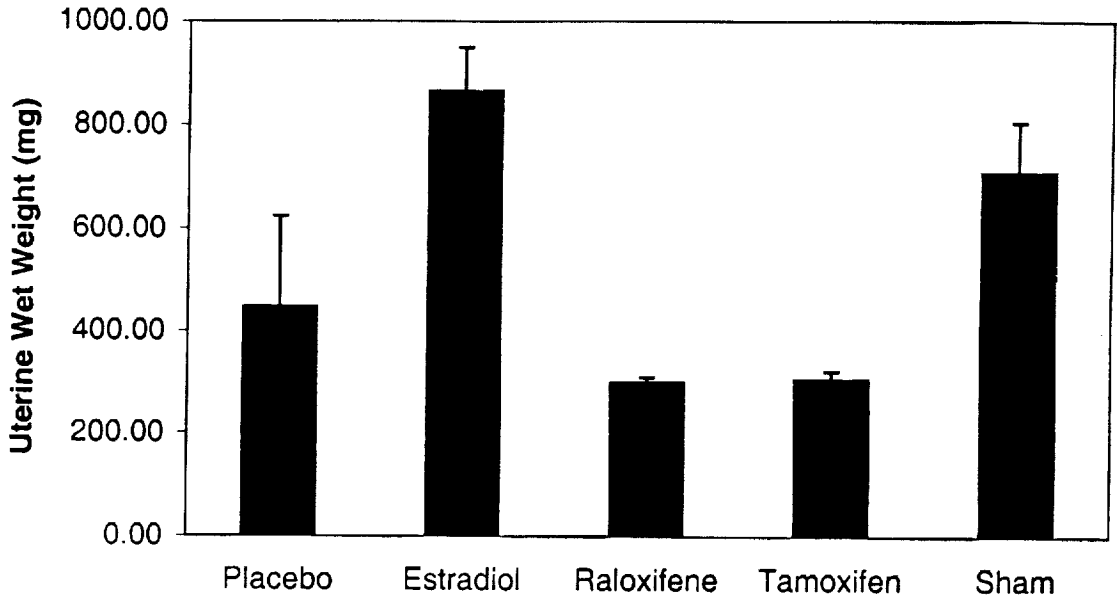


Figure 15: The SERMs, raloxifene and tamoxifen, reduced uterine wet weights in ovariectomized female rats. Six-month old female Sprague Dawley rats were ovariectomized and immediately implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Animal were sacrificed, uteri dissected out and wet weights obtained. Values represent the mean ± SEM of n=7-8 animals per group.

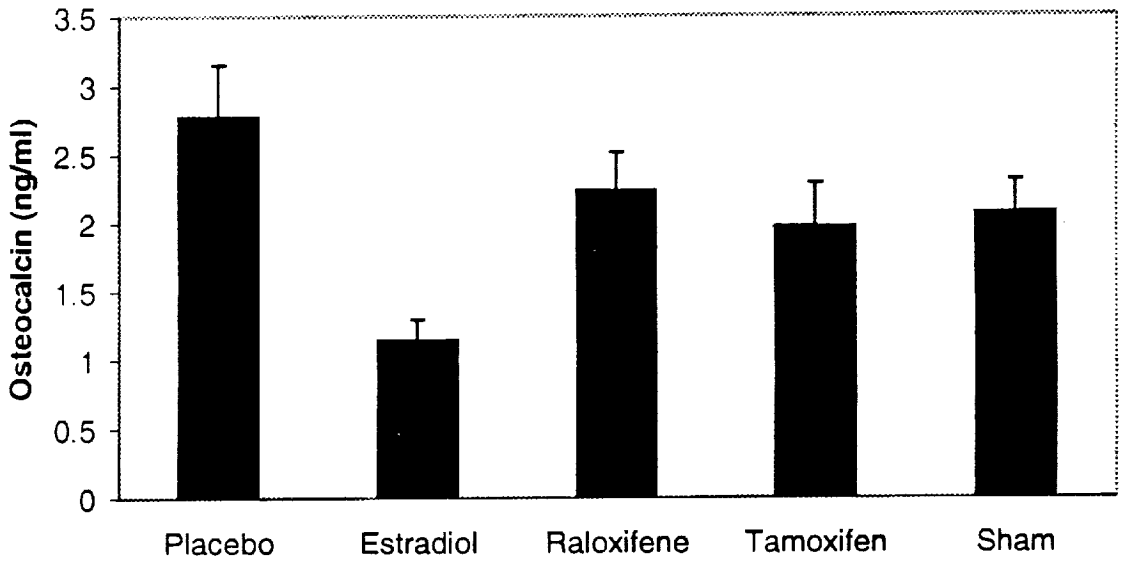


Figure 16: Effect of the SERMs, raloxifene and tamoxifen, in comparison to 17β-estradiol, on serum osteocalcin levels in ovariectomized female rats. Six-month old female Sprague Dawley rats were ovariectomized and immediately implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Animals were sacrificed, blood collected for serum preparation and osteocalcin values measured by EIA. Values represent the mean ± SEM of n=7-8 animals per group.

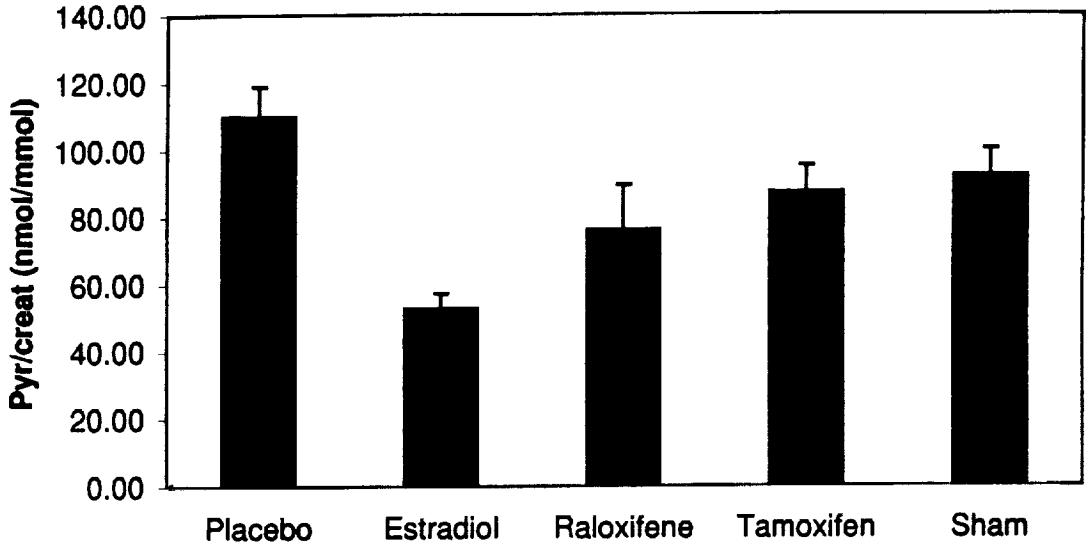


Figure 17: Effect of the SERMs, raloxifene and tamoxifen, in comparison to 17β-estradiol, on urinary pyridinium crosslink levels in ovariectomized female rats. Six-month old female Sprague Dawley rats were ovariectomized and immediately implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Urine samples were collected for the day proceeding sacrifice. Pyridinium crosslink values were measured by EIA and standardized to urinary creatinine levels. Values represent the mean ± SEM of n=7-8 animals per group.

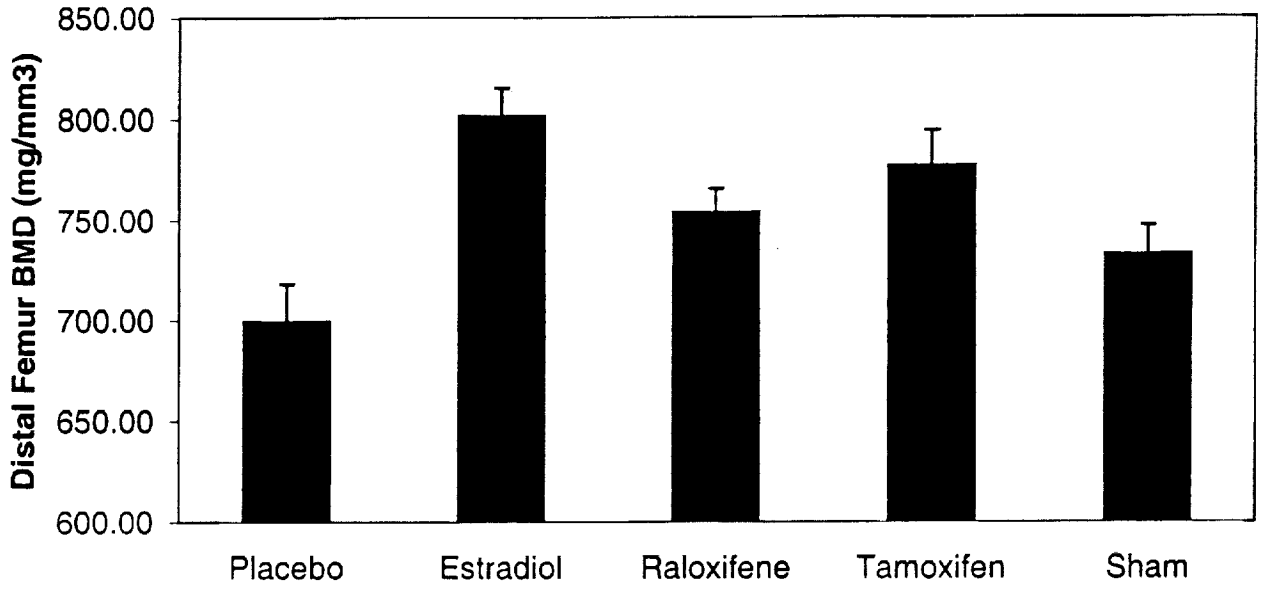


Figure 18: Effect of the SERMs, raloxifene and tamoxifen, in comparison to 17β-estradiol, on bone mineral density of the distal femur in ovariectomized female rats. Six-month old female Sprague Dawley rats were ovariectomized and immediately implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Animals were sacrificed and left femurs dissected out for *ex vivo* pQCT measurements of bone mineral density taken 5, 5.5 and 6 mm from the most distal aspect. Average values were calculated. Values represent the mean ± SEM of n=7-8 animals per group.

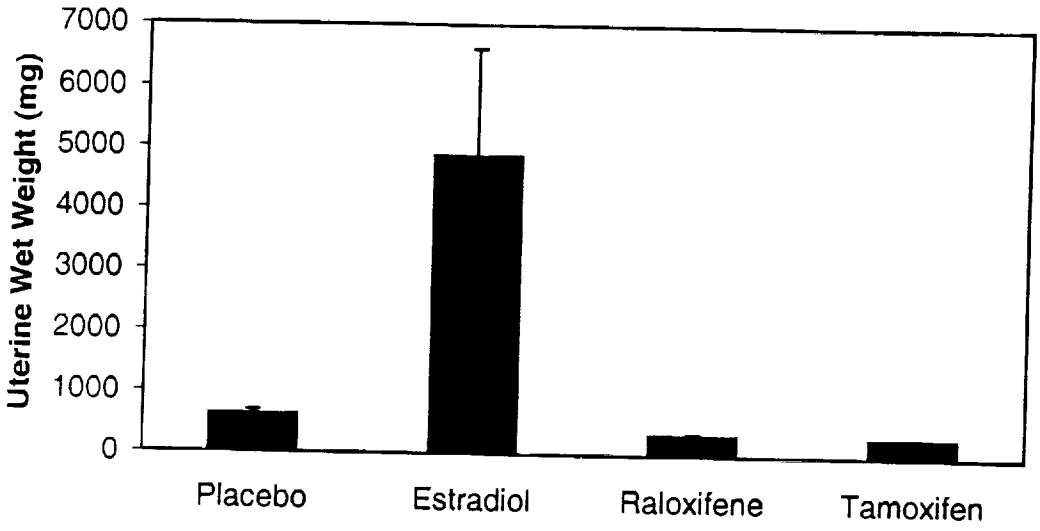


Figure 19: The SERMs, raloxifene and tamoxifen, reduced uterine wet weights in ovary-intact female rats. Six-month old female Sprague Dawley rats were implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Animal were sacrificed, uteri dissected out and wet weights obtained. Values represent the mean ± SEM of n=7-8 animals per group.

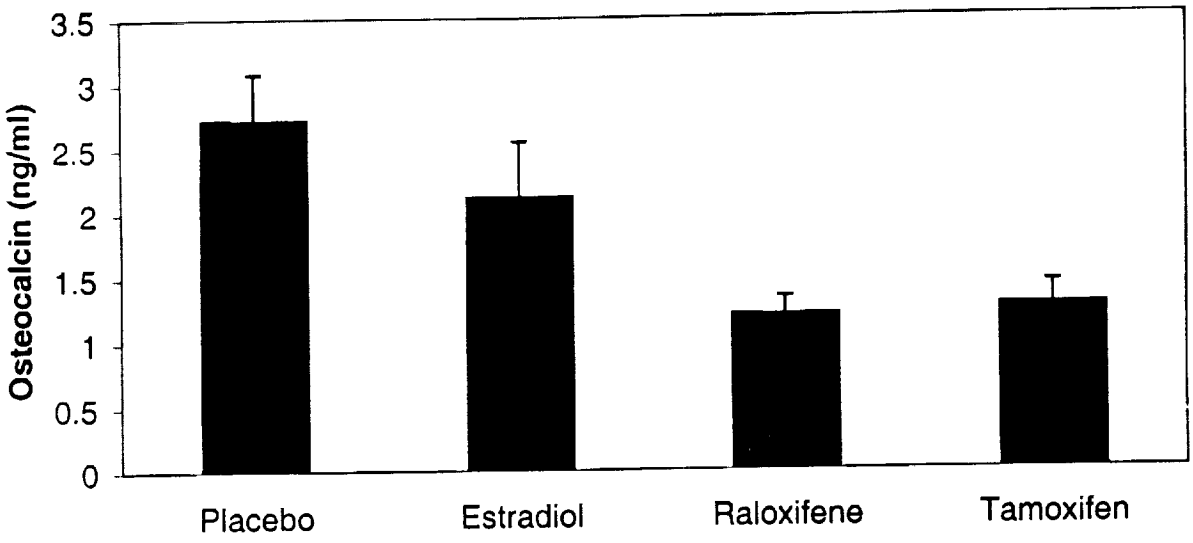


Figure 20: Effect of the SERMs, raloxifene and tamoxifen, in comparison to 17β-estradiol, on serum osteocalcin levels in ovary-intact female rats. Six-month old female Sprague Dawley rats were implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Animals were sacrificed, blood collected for serum preparation and osteocalcin values measured by EIA. Values represent the mean ± SEM of n=7-8 animals per group.

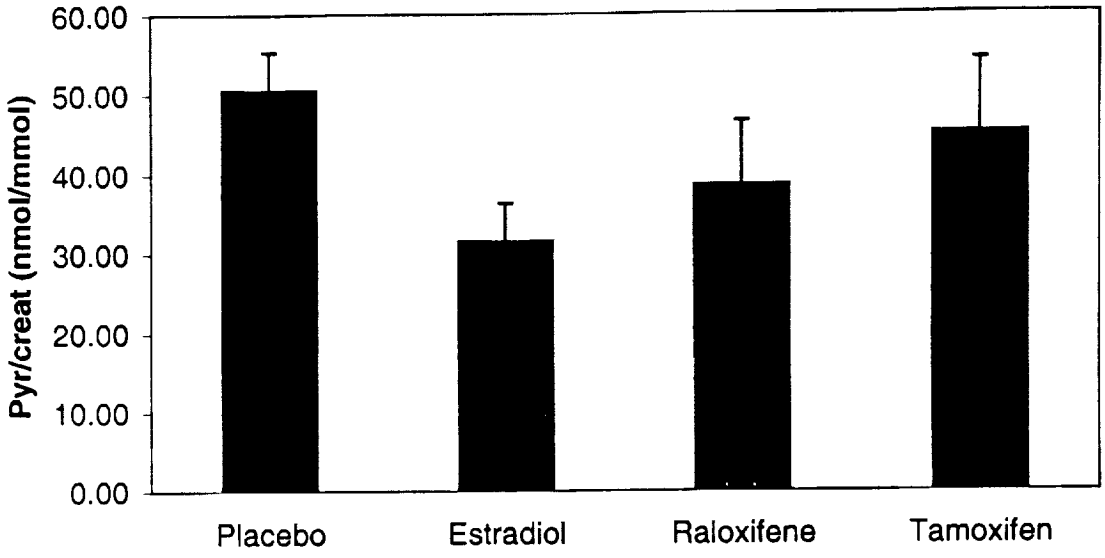


Figure 21: Effect of the SERMs, raloxifene and tamoxifen, in comparison to 17β-estradiol, on urinary pyridinium crosslink levels in ovary-intact female rats. Six-month old female Sprague Dawley rats were implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Urine samples were collected for the day proceeding sacrifice. Pyridinium crosslink values were measured by EIA and standardized to urinary creatinine levels. Values represent the mean ± SEM of n=7-8 animals per group.

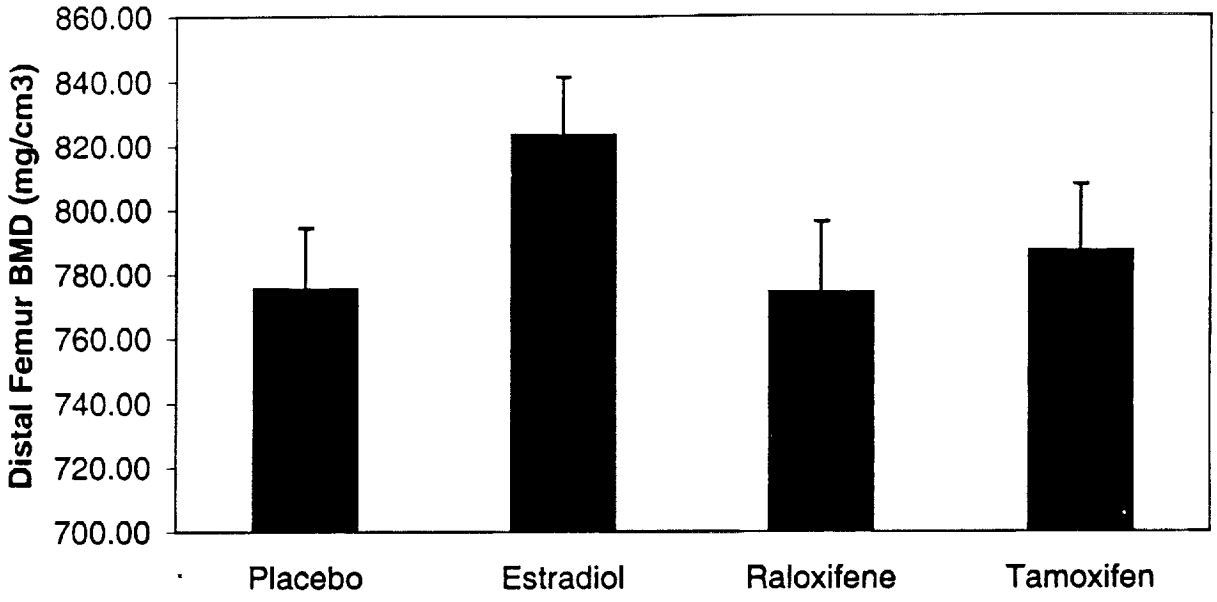


Figure 22: Effect of the SERMs, raloxifene and tamoxifen, in comparison to 17β-estradiol, on bone mineral density of the distal femur in ovary-intact female rats. Six-month old female Sprague Dawley rats were implanted with time release pellets designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene, 583 μg/day tamoxifen or placebo over a 28 day period. Animals were sacrificed and left femurs dissected out for *ex vivo* pQCT measurements of bone mineral density taken 5, 5.5 and 6 mm from the most distal aspect. Average values were calculated. Values represent the mean ± SEM of n=7-8 animals per group.

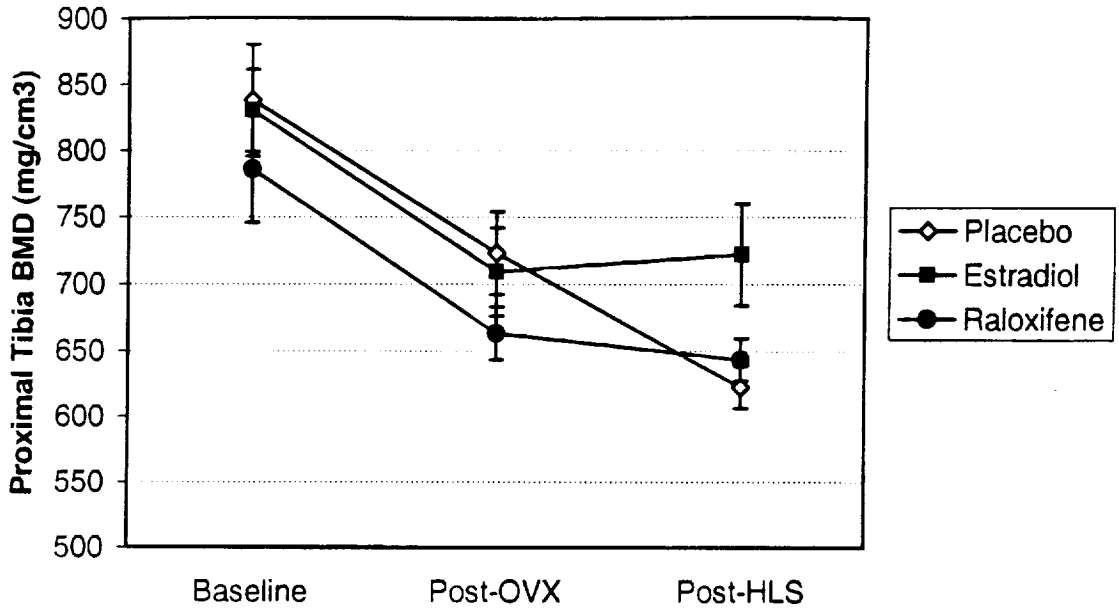


Figure 23: Raloxifene or 17β-estradiol prevent hindlimb suspension-induced bone loss at the proximal tibia. Five-month old female rats were ovariectomized and allowed to recover for 28 days. At six months of age, rats were implanted with a time release pellet designed to deliver 12 μg/day 17β-estradiol, 535 μg/day raloxifene or placebo over a 28 day period, and subjected to 28 days of hindlimb suspension. Measurements of proximal tibia bone mineral density were obtained on animals prior to ovariectomy (baseline), immediately prior to time release pellet insertion and initiation of hindlimb suspension (Post-Ovx) and after 28 days of hindlimb suspension (Post-HLS). Values represent the mean ± SEM for placebo (n=3), estradiol (n=4) and raloxifene (n=4) treated animals.

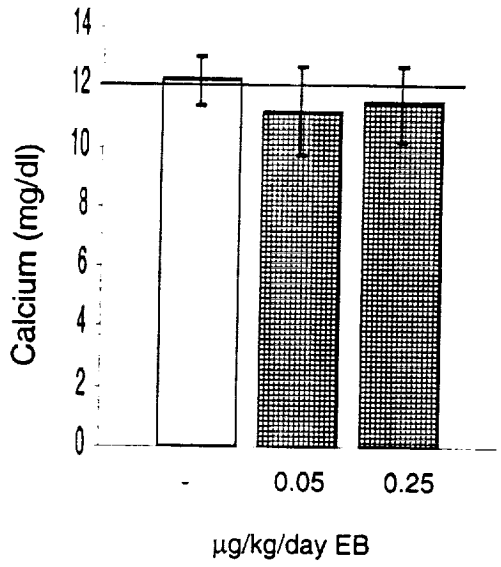


Figure 24: EB1089 is Normocalcemic in Sprague Dawley rats: Sprague Dawley rats were treated with vehicle (open bars), 0.05 µg/kg/day EB1089 or 0.25 µg/kg/day EB1089 (hatched bars) for 28 days. The normal level for serum calcium is shown by the line across the figure.

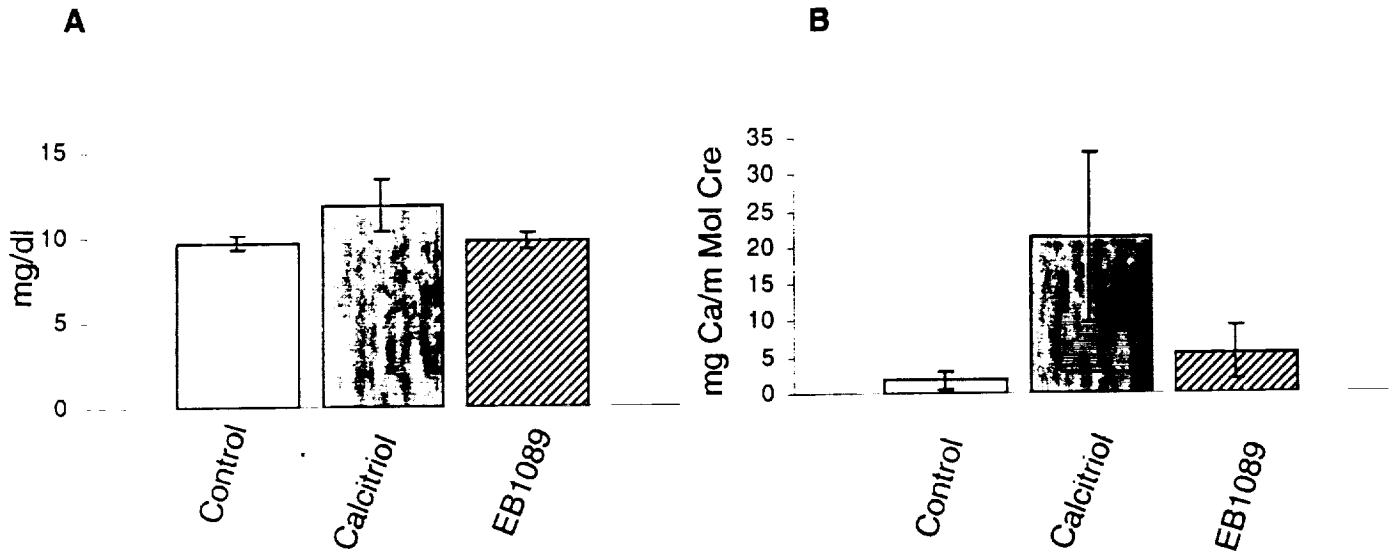


Figure 25: EB1089 does not increase serum or urine calcium in hind limb suspended rats. Hind limb suspended Sprague Dawley rats were treated with vehicle or calcitriol or EB1089 for 28 days and the serum (A) and urine calcium (B) were analyzed. The urine calcium levels were normalized to the urine creatinine levels.

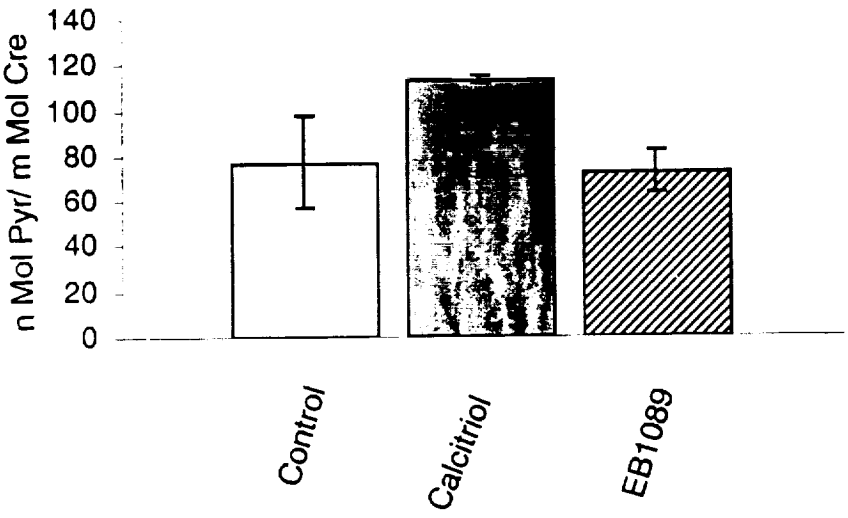


Figure 26: Effect of calcitriol and EB1089 treatment on urine Pyridinium cross links in hind limb suspended rats. Hind limb suspended rats were treated with vehicle, calcitriol or EB1089 for 28 days. The urine pyridinium levels were normalized to the urine creatinine.

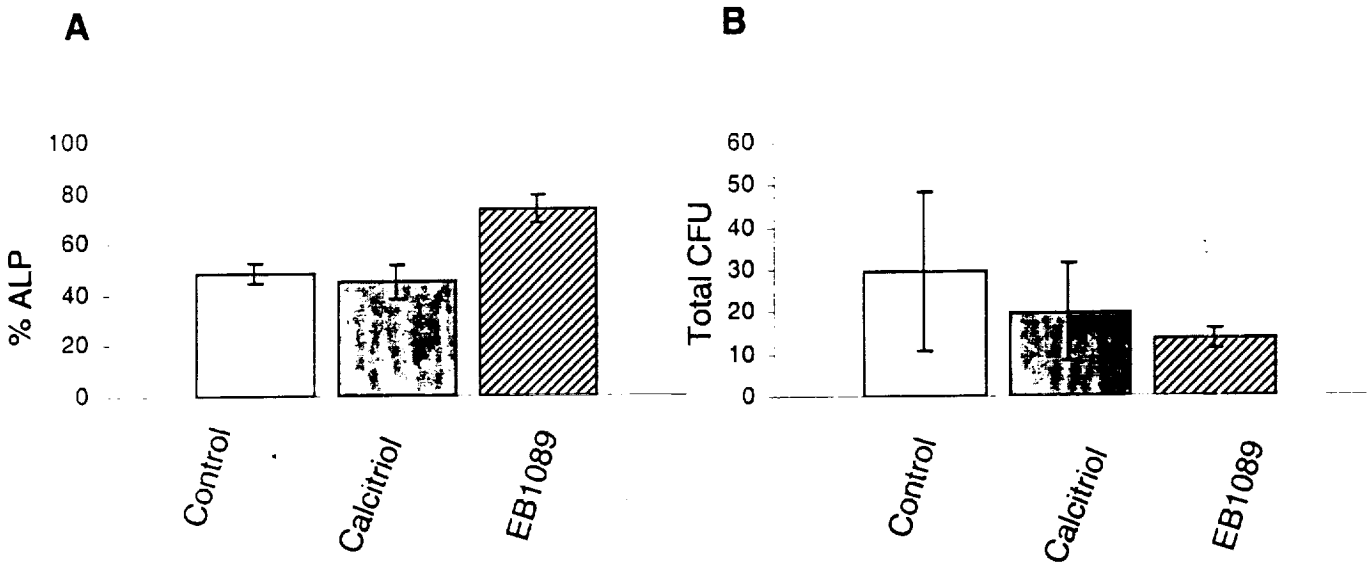


Figure 27: Effect of Calcitriol or EB1089 on primary bone marrow cultures from hind limb suspended rats. Bone marrow from hind limb suspended rats treated with vehicle, calcitriol or EB1089 were cultured for 10 days and stained for Alkaline Phosphatase and the number of colonies counted. A. Percent Alkaline Phosphatase positive colonies (ALP) and B. Total Colony forming unit fibroblasts (CFU-F).

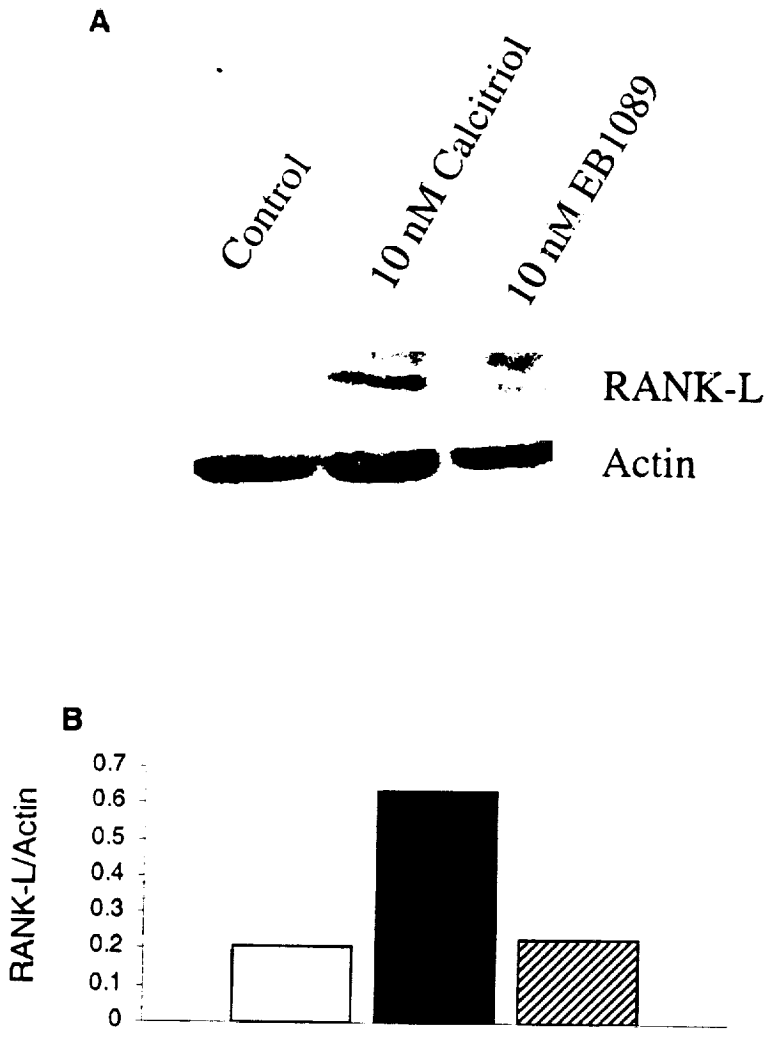


Figure 28: EB1089 does not induce RANK-Ligand Protein *in vitro*. Primary bone marrow cells were treated *in vitro* with vehicle, 10 nM calcitriol or 10 nM EB1089 and the protein were immunoblotted with RANK-L antibody. A. Western showing RANK-L. B. Densitometric quantitation of the protein from the western blot.

Table I: Effects of EB1089 on Proximal Tibia of Male Rats Hindlimb Suspended for 28 days.

Pre-Post pQCT results for Proximal Tibia.

Data was obtained on day 0 and 28. Proximal tibia was scanned at 5, 5.5, 6mm from protimal plateau of the right leg. The three slices from each animal were averaged to get a single representative value. Statistics run on SAS 6.12. Two-way analysis of variance (group and time) was used for all variables with repeated measures on time. When an overall significant interaction of group and time was found ($p < 0.05$) simple main effect analysis was utilized to determine which groups were significantly different. Results are below (*denotes significantly ($p< 0.05$) compared to pre value):

VARIABLE	GROUP*TIME p Value
BMC (mg/mm)	0.0016
Total BMD (mg/cm^3)	0.0001
Cortical BMD (mg/cm^3)	0.1809
Trabecular BMD (mg/cm^3)	0.0001
Total Area (mm^2)	0.7708
Marrow Area (mm^2)	0.2162
Cortical Area (mm^2)	0.0513

BMC	PRE	POST
Vehicle	11.48 ± 0.19	10.34 ± 0.4*
EB1089	12.0 ± 0.40	11.91 ± 0.55
VitD	11.66 ± 0.30	12.6 ± 0.35*

Bone Mineral Content: No difference in groups at baseline. Vehicle animals significantly decreased, while VitD animals significantly increased. No change in EB1089 group.

Total Bone Mineral Density: No difference in groups at baseline. Vehicle animals significantly decreased, while VitD animals significantly increased. No change in EB1089 group.

Total BMD	PRE	POST
Vehicle	642 ± 4.5	586 ± 14.8*
EB1089	640 ± 11.2	613 ± 13.5
VitD	636 ± 12.7	693 ± 11.6*

Cortical Bone Mineral Density: No significant difference in groups at baseline. No group*time effect.

Cortical BMD	PRE	POST
Vehicle	1128 ± 2.6	1122 ± 20.6
EB1089	1106 ± 15.7	1114 ± 17.5
VitD	1100 ± 6.7	1141 ± 12.2

Trabecular Bone Mineral Density: No significant difference in groups at baseline. Vehicle animals significantly decreased, while VitD animals significantly increased. No change in EB1089 group.

Trabecular BMD	PRE	POST
Vehicle	207 ± 8.6	172 ± 8.3*
EB1089	214 ± 22.9	206 ± 23.8
VitD	218 ± 11.2	265 ± 14.3*

Total Area: No significant difference in groups at baseline. No significant group * time effect.

Total Area	PRE	POST
Vehicle	17.9 ± 0.29	18.4 ± 0.86
EB1089	18.9 ± 0.87	19.5 ± 1.18
VitD	18.4 ± 0.53	18.2 ± 0.52

Marrow Area: No significant difference in groups at baseline. No significant group * time effect.

Marrow Area	PRE	POST
Vehicle	9.5 ± 0.22	10.5 ± 0.69
EB1089	10.0 ± 0.65	10.9 ± 0.90
VitD	9.7 ± 0.48	9.25 ± 0.40

Cortical Bone Area: No significant difference in groups at baseline. Vehicle animals significantly decreased, while VitD and EB1089 groups were not significantly changed.

Cortical Area	PRE	POST
Vehicle	8.4 ± 0.13	7.9 ± 0.21*
EB1089	8.9 ± 0.25	8.6 ± 0.32
VitD	8.7 ± 0.16	8.9 ± 0.20

SUMMARY: Area does not appear to be affected in any groups after 28d HU. Vehicle animal data suggests the possibility of endocrotical resorption due to a decreased cortical area and trend toward increasing marrow area. EB1089 and VitD are unchanged. With no difference in Cortical BMD, the change in Total BMD is accounted for primarily by similar changes in Trabecular BMD. BMD change without change in area accounts for the change in BMC in both vehicle and VitD animals.

Pre-Post pQCT results for Mid-diaphysisTibia.

Data was obtained on day 0 and 28. Mid-diaphysis tibia was scanned at 50% of the total right leg length (as measured in the scout view of the pQCT). One slice was taken. Statistics were run on SAS 6.12. Two-way analysis of variance (group and time) was used for all variables with repeated measures on time. There were no significant
group * time interactions at this site. Data on pre/post mean ± SE is presented below:

Mid-Diaphysis Tibia pQCT Mean ± SE							
	Vehicle		**EB1089**		**VitD**		**Group*time p value**
	Pre	Post	Pre	Post	Pre	Post	
BMC (mg/mm)	8.1 ± 0.14	8.2 ± 0.13	8.35 ± 0.16	8.51 ± 0.15	8.14 ± 0.20	8.36 ± 0.19	0.3381
BMD (mg/cm^3)	1311 ± 5.2	1325 ± 9.1	1292 ± 17	1325 ± 9.8	1294 ± 14	1334 ± 8.8	0.3952
Cortical Area (mm^2)	6.18 ± 0.12	6.20 ± 0.10	6.46 ± 0.16	6.42 ± 0.13	6.31 ± 0.20	6.27 ± 0.12	0.8054
Total Area (mm^2)	8.64 ± 0.14	8.71 ± 0.14	9.06 ± 0.29	9.17 ± 0.31	8.87 ± 0.24	8.85 ± 0.19	0.7392
Marrow Area (mm^2)	2.28 ± 0.07	2.42 ± 0.09	2.47 ± 0.15	2.66 ± 0.22	2.41 ± 0.06	2.48 ± 0.10	0.4779
Cortical Thickness (mm)	0.818 ± 0.12	0.922 ± 0.01	0.947 ± 0.02	0.905 ± 0.02	0.948 ± 0.02	0.770 ± 0.15	0.2539
$CSMI_x$ (mm^4)	6.62 ± 0.25	6.93 ± 0.22	6.81 ± 0.41	7.46 ± 0.47	6.67 ± 0.35	7.03 ± 0.28	0.6890
$SecMod_x$ (mm^4)	3.39 ± 0.10	3.51 ± 0.10	3.20 ± 0.16	3.75 ± 0.16	3.28 ± 0.16	3.63 ± 0.08	0.5164

6B. Publications, Abstracts and Manuscripts

1. Yamaguchi T, Kifor O, Chattopadhyay N, Brown EM. (1998) Expression of extracellular calcium (Ca^{2+}_o)-sensing receptor in the clonal osteoblast-like cell lines, UMR-106 and SAOS-2. Biochem Biophys Res Commun; 243:753-757.

2. Yamaguchi T, Chattopadhyay N, Butters RR, Kifor O, Brown EM. (1998) Expression of Extracellular Calcium (Ca^{2+}_o)-Sensing Receptor in Human Peripheral Blood Monocytes. Biochem Biophys Res Commun; 246:501-506.

3. Yamaguchi T, Chattopadhyay N, Kifor O, Brown EM. (1998) Extracellular Calcium (Ca^{2+}_o)-Sensing Receptor in a Mouse Bone Marrow-Derived Stromal Cell Line (ST2): Potential Mediator of the Actions of Ca^{2+}_o on the Function of ST2 Cells. Endocrinology; 139:3561-3568.

4. Yamaguchi T, Kifor O, Chattopadhyay N, Bai M, Brown EM. (1998) Extracellular calcium (Ca^{2+}_o)-sensing receptor in a mouse monocyte-macrophage cell line (J774): Potential mediator of the actions of Ca^{2+}_o on the function of J774 cells. J Bone Miner Res; 13:1390-1397.

5. Yamaguchi T, Chattopadhyay N, Kifor O, Butters Jr R, Sugimoto T, Brown E. (1998) Mouse Osteoblastic Cell Line (MC3T3-E1) Expresses Extracellular Calcium (Ca^{2+}_o)-Sensing Receptor and Its Agonists Stimulate Chemotaxis and Proliferation of MC3T3-E1 cells. J Bone Miner Res; 13:1530-1538.

6. Narayanan R, Smith CL and Weigel NL: EB1089 treatment partially reverses the reduction in vitamin D receptor activity in MG-63 cells subjected to simulated microgravity. 21st Annual meeting of the American Society for Bone and Mineral Research, 1999. St. Louis, MO. September 30-October 4, 1999 (Abstract).

7. Yamaguchi T, Chattopadhyay N, Brown EM. (2000) G Protein-Coupled Extracellular Ca^{2+} (Ca^{2+}_o)-Sensing Receptor (CaR): Roles in Cell Signaling and Control of Diverse Cellular Functions. Adv Pharmacol 47: 209-253.

8. Narayanan R, Smith CL and Weigel NL. Vector-averaged gravity alters cell signaling and decreases vitamin D receptor activity in MG-63 cells. Submitted, 2000.

9. Narayanan R and Weigel NL. Cell specific interactions between 1,25(OH)2 D3 and mitogen activated protein kinase (MAPK) signaling pathways. Eleventh Vitamin D workshop, Nashville, TN, 2000 (Abstract).

10. Smith CL, Nolan C, Lin J and Parker CL: Regulation of osteoblast function by estradiol and selective estrogen receptor modulators (SERMs) in differentiating MC3T3-E1 cells. American Society for Bone and Mineral Research, 22nd Annual Meeting, Toronto, Canada. Abstract #____, September 22-26, 2000. (Abstract).

11. Narayanan R, Gaddy-Kurten D, Montague DL, Smith CL and Weigel NL: EB1089 preferentially stimulates osteoblastogenesis in bone marrow culture of Sprague Dawley rats. American Society for Bone and Mineral Research, 22nd Annual Meeting, Toronto, Canada. Abstract #____, September 22-26, 2000 (Abstract).

12. Ye C-P, Yamaguchi T, Chattopadhyay N, Sanders JL, Vassilev PM, Brown EM. (2000) Extracellular Calcium-Sensing Receptor (CaR)-Mediated Opening of an Outward K^+

Channel in Murine MC3T3-E1 Osteoblastic Cells: Evidence for Expression of a Functional CaR. Bone; 27:21-27.

13. Olszak I, Poznansky MC, Kos C, Pollak M, Brown EM, Scadden DT. (2000) Extracellular calcium is an ionic chemokine for human immune cells. J. Clin Invest;105:1299-1305.

14. Yamaguchi T, Ye C, Chattopadhyay N, Vassilev P, Brown EM. (2000) Enhanced Expression of extracellular calcium (Ca^{2+}_o)-sensing receptor in monocyte-differentiated HL-60 cells: potential role in regulation of cellular proliferation and a nonselective cation channel. Calcif Tiss Int; 66:375-382.

15. Yamaguchi T, Chattopadhyay N, Kifor O, Ye C, Vassilev PM, Sanders JL, Brown EM. Functional Expression of the Extracellular Calcium (Ca^{2+}_o)-Sensing Receptor (CaR) in the Human Osteoblastic MG-63 Cell Line. Am J Physiol (Cell Physiol) (in press).

FINAL REPORT
To
NSBRI

BONE BLOOD FLOW DURING SIMULATED MICROGRAVITY: PHYSIOLOGICAL AND MOLECULAR MECHANISMS

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NSBRI Grant NCC9-58-H

FINAL REPORT:
BONE BLOOD FLOW DURING SIMULATED MICROGRAVITY: PHYSIOLOGICAL AND MOLECULAR MECHANISMS

EXECUTIVE SUMMARY

Blood flow to bone has been shown to affect bone mass and presumably bone strength. Preliminary data indicate that blood flow to the rat femur decreases after 14 days of simulated microgravity, using hindlimb unloading (HU). If adult rats subjected to HU are given dobutamine, a synthetic catecholamine which can cause peripheral vasodilation and increased blood flow, the loss of cortical bone area usually observed is prevented. The primary aim of this project is to characterize changes in 1) bone blood flow, 2) indices of bone mass, geometry, and strength, and 3) changes in gene expression for candidate genes for mechanotransduction in bone after 3, 7, 14, 21, and 28 days of HU in the adult rat. Using a rat of at least 5 months of age avoids inadvertently studying effects of simulated microgravity on growing, rather than adult, bone.

The key contributions of these studies lie in the quantification of blood flow to various compartments of bone and marrow with the acute adjustment to the tail suspension posture (after 10 minutes) and after 7 and 28 days of tail suspension. Ten minutes of HU significantly decreases flow to the proximal femur, the femoral shaft and to femoral shaft marrow. By 7 days of HU, blood flow to all portions of the femur, including distal metaphysis, is sigificantly less than flow to these regions in cage-activity control rats. These decreases in flow are due to an increase in vascular resistance; this increased resistance is maintained through 28 days of HU. Conversely, blood flow to bone of the forelimb and head (skull, mandible) is increased during HU with the acute shift to the tail suspension posture. However, blood flow returns to normal with 7 days HU due to compensatory increases in vascular resistance. These alterations in blood flow were accompanied by changes in bone mass, with those bones experiencing lower flows during HU decreasing in mass (femur, tibia), whereas those bones experiencing that acute increase in flow with HU increasing in mass (mandible, clavicle, humerus).

Because radiolabeled microspheres are used to measure blood flow, rendering all tissues radioactive, bones from these animals could not be further processed. Separate experiments were performed to document the time course of alterations in bone mineral content, bone geometry, mechanical properties and gene expression with HU. Histomorphometric analyses of fluorochrome-labeled bone reveal large decrements in mineral apposition rate and bone formation rate at the tibio-fibular junction (60% and 90% declines, respectively) in these mature adult rats. Our data confirm that of earlier investigators (Dehority et al., *Amer. J. Physiol.*, 1999) in that decreases in bone formation are slower to develop in the mature rat skeleton as opposed to the better characterized response of young growing rats, but more prolonged.

Few changes were noted in bone mineral density (BMD) of mid-shaft cortical bone. Alterations in tibial BMD and cross-sectional area appear to parallel growth-related changes in the humerus. Not surprisingly, mechanical properties at this site were not affected either. Analyses using pQCT of the proximal tibial metaphysis reveal a compartment-specific alterations in BMD, where cancellous (trabecular) bone is decreasing in BMD even as the cortical shell gains in BMD during 28 d HU. Novel analyses of cancellous bone mechanical properties reveal a decline in both elastic modulus and ultimate stress in this core of the proximal tibial metaphysis after 28 d HU. Clearly, mechanical strength of bone is compromised at sites rich in cancellous bone in the unloaded limb, even in the mature adult skeleton in which growth processes are minimal.

Gene expression is altered in proximal femur samples from these unloaded rats. Early changes at 3 days reveal an increased expression of cytokines favoring bone resorption (interleukin-6 and interferon-γ) and a decrease in expression of TGF-β1, which normally stimulates bone formation activity. These alterations remain to be confirmed in longer duration studies. Microarray data indicate upregulation of a number of genes potentially important in regulating bone cell activity: integrin abv3 and abv5, nitric oxide synthase, prostaglandin synthase 1 and 2. A down-regulation of several oncogenes (fos, abl) was noted, as well as for BMP receptors types I and II. A very recent finding with micro-array analysis points to an upregulation of a membrane kinase previously unidentified in bone in proximal femurs from rats subjected to 28d HU.

These results have important implications for the development of countermeasures to ameliorate bone loss with prolonged exposure to weightlessness. We have established a time course for declines in blood flow to bone, using state-of-the-art microsphere studies, which precede reductions in bone formation (at mid-shaft tibia) and BMD and mechanical propertiesof cancellous bone in the proximal tibia. If,indeed, continuing experiments can demonstrate a causal link between altered blood flow and shifts in bone remodeling activity, a whole new category of countermeasures might be considered. Some pharmacological agents may effect a general vasodilation and therefore increase in blood flow, but relatively benign physical measures [heating, exercise, lower body negative pressure (with or without exercise)] can be tested for effectiveness in earth-based studies in increasing blood flow to bone. These data on mature adult rats, a better model for adult human bone than the more widely used growing rat, also suggest that sites rich in cancellous bone (proximal femur, proximal tibia, distal femur) in unloaded limbs are at the highest risk for fracture with prolonged exposure to microgravity. We may have over-estimated the rate of change in cortical bone BMD and cortical bone geometry in the past having relied on information from rapidly growing young rats. Our gene expression data provide some mechanistic data on which to build future countermeasure strategies, given early changes in regulatory peptides important in regulating bone cell activity.

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PROJECT RESEARCH ACTIVITY—
BONE BLOOD FLOW DURING SIMULATED MICROGRAVITY: PHYSIOLOGICAL AND MOLECULAR MECHANISMS

The following Specific Aims and hypotheses were proposed in this grant. Following each of the Specific Aims, a manuscript/abstract reference(s) is given which addressed this aim. Other original research publications acknowledging support from this grant are listed in Appendix III.

Specific Aim I. *To characterize temporal changes in bone blood flow, as well as indices of bone mass, geometry, and strength after 7, 14, 21 and 28 days of hindlimb suspension in the adult rat. Responses in the unloaded hindlimb bones (tibial shaft, femoral neck) will be compared with those in the weightbearing humerus and the non-weightbearing calvarium.*

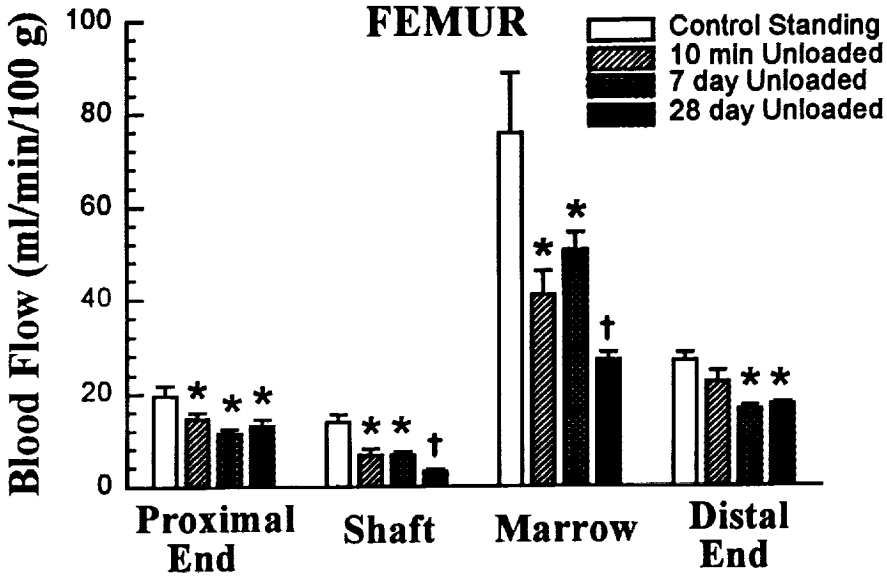
1. Colleran, P.N., M.K. Wilkerson, S.A. Bloomfield, L.J. Suva, R.T. Turner, and M.D. Delp. Alterations in skeletal perfusion with simulated microgravity: A possible mechanism for bone remodeling. *J. Appl. Physiol.* 89: 1046-1054, 2000.

2. Delp, M.D., P.N. Colleran, M.K. Wilkerson, E.P. Lorenz, M.R. Allen, H.A. Hogan, L.J. Suva, and S.A. Bloomfield. Short-term hindlimb unloading diminishes bone mineral density, area and blood flow in rats (Abstract). *J. Bone Min. Res.* 14:S315, 1999.

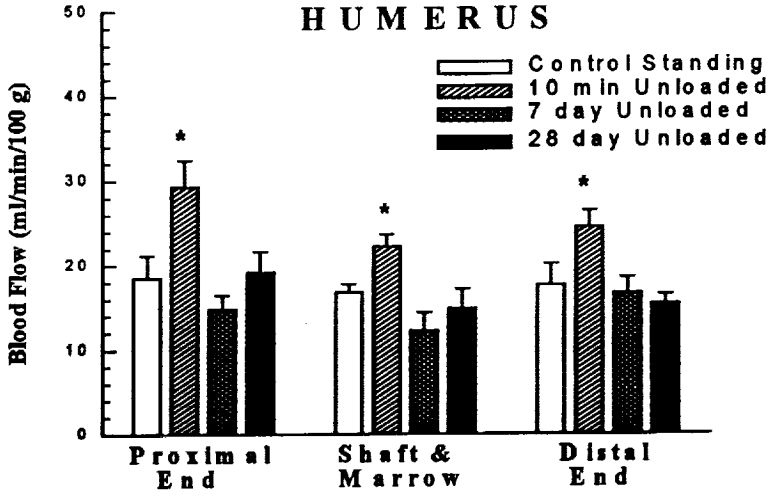
The primary purpose of this aim was to determine whether the unloading of hindlimb bones and the corresponding cephalic fluid shift alter bone perfusion rates. We hypothesized perfusion would be diminished in the hindlimb bones and increased in skeletal structures of the forelimb and head. Furthermore, we also hypothesized that reductions in blood flow to the hindlimb bones would occur in regions where unloading-induced bone loss occurs.

The design of the experiments as originally proposed had to be altered because of the unexpected rapidity in which bone and marrow perfusion were altered by unloading. Blood flow was therefore measured in these bones after 10 min, 7 days and 28 days of unloading. The results indicate that hindlimb unloading rapidly (within 10 min) diminishes blood flow to the femoral and tibial metaphysis (cancellous bone), diaphysis (cortical bone) and marrow, and prolonged unloading ($\geq$ 7 days) further decreases perfusion of the femoral shaft and marrow. Chronic unloading was also necessary to decrease fibular perfusion rate.

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In contrast, hindlimb unloading acutely elevates bone blood flow to the forelimb and head of the rat, i.e., the humerus, clavicle, skull and mandible.



The decline in blood flow to the hindlimb bones appears to coincide with a diminished mass of the femur and tibia. Correspondingly, the acute increase in blood flow to forelimb, shoulder and head bones appears to coincide with reports of increased bone mass in HU rats (e.g., see **Table 1 below**).

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Table 1. Bone mass in control and hindlimb unloaded (HU) rats.

	Control	7 Days	28 Days	
		HU		HU
n	11	11		7
Femur	1,490 ± 50	1,410 ± 31		1,330* ± 20
Tibia	1,025 ± 25	1,052 ± 30		962^{+} ± 15
Fibula	124 ± 9	107 ± 9		95^{+} ± 8
Pelvis	2,330 ± 89	2,500 ± 87		2,320 ± 80
Humerus	596 ± 21	601 ± 19		648^{+} ± 16
Radius/Ulna	509 ± 21	515 ± 31		517 ± 20
Scapula	466 ± 41	515 ± 41		502 ± 46
Clavicle	74 ± 4	77 ± 4		87* ± 6
Skull	3,550 ± 167	3,890 ± 166		3,930 ± 164
Mandible	1,480 ± 44	1,540 ± 39		1,640* ± 30

Values are means ± SE. Units for mass are mg. *Mean value significantly different from that of control rats ($P<0.05$); $^{+}$mean value different from that of control animals ($P<0.1$).

Histomorphometric analysis of fluorochrome-labeled tibiae reveals that it takes several weeks of HU for a slowing of mineral apposition and bone formation rates to be detected in our mature adult rats (**Table 5** and **Figure 3, Appendix I**). After 21 days of HU significant 60% and 90% declines in mineral apposition rate and periosteal bone formation rate, respectively, occur. These data are quite similar to those of Dehority et al. (Amer. J. Physiol. 276: E62-E69, 1999), who also used this mature adult model (6-mo-old males), except that those investigators found significant declines in these variables after 14 days HU in mid-shaft tibial bone. Hence, one of our significant objectives has been fulfilled: we have demonstrated a decline in blood flow to bone which **precedes** a change in bone cell activity, as reflected by these measures of osteoblast activity.

As might be expected in the mature adult rat in whom longitudinal and radial growth is much diminished as compared to young growing rats, these changes in periosteal MAR and BFR don't immediately translate into detectable changes in bone structure. *Ex vivo* pQCT measures of bone

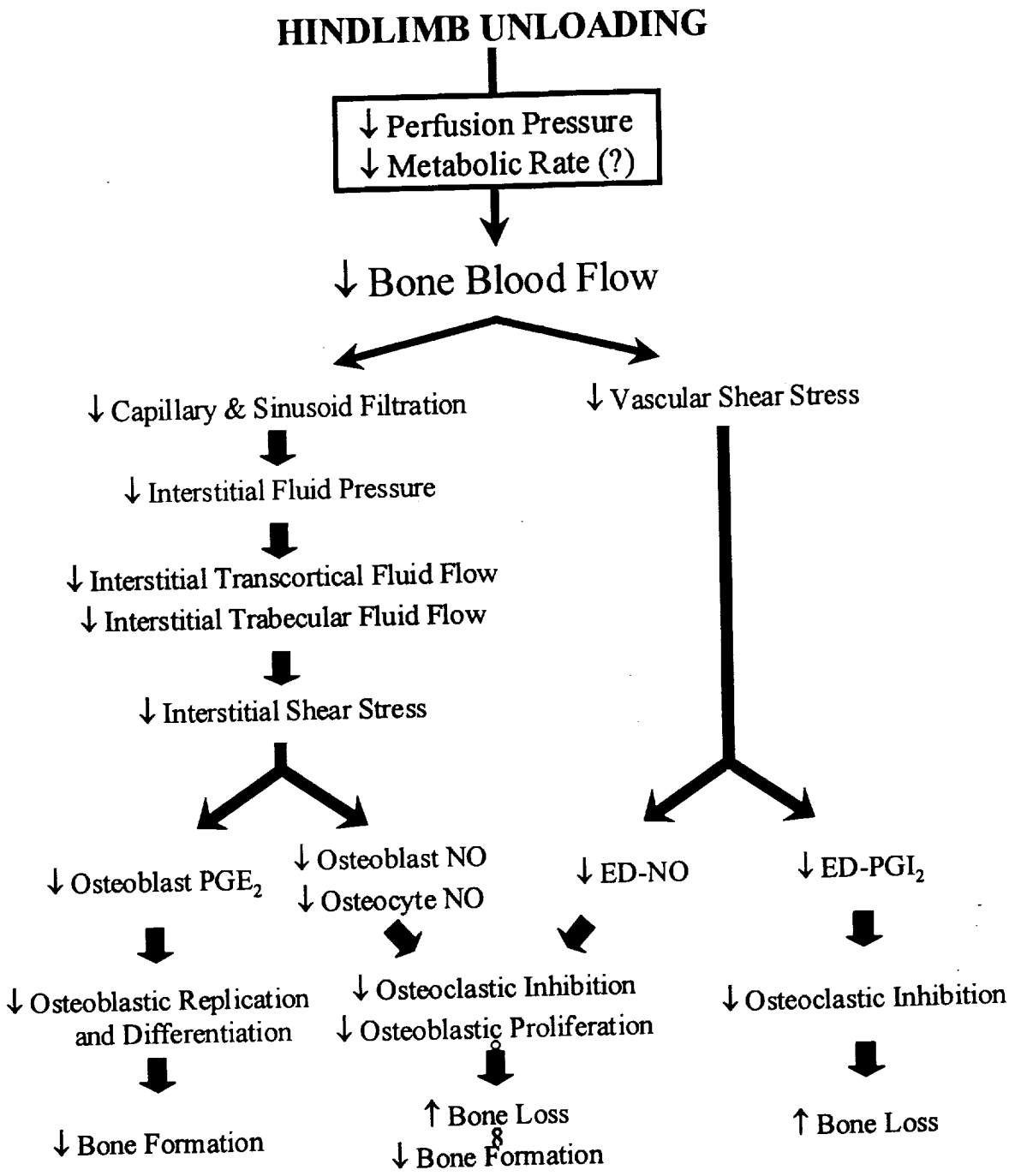
geometry and bone mineral density reveal no measurable effect of hindlimb unloading on the tibial shaft; indeed, BMD of the mid-shaft cortical bone continues to increase over 28 days to a similar magnitude as observed in the aging controls (**Figure 2,** Appendix I). However, interesting shifts in BMD of the trabecular and cortical compartments are noted in the proximal tibial metaphysis over 28 days of hindlimb unloading (**Figure 1,** Appendix I). The BMD of the cortical shell increases in hindlimb unloaded rats to a similar degree as it does in aging controls, but the BMD of the trabecular core declines by 23% as compared to the baseline controls. Future studies will center on histomorphometric measures of trabecular bone to verify declines in MAR and BFR at this site, as has been observed in younger rats subjected to spaceflight and tail suspension.

Testing of mechanical properties of bone reveal that bone site's propensity to fracture. As revealed in **Table 4** (Appendix I), few changes were noted in structural or material properties of mid-shaft cortical bone in unloaded tibiae. This is not unexpected given the lack of change in bone area and BMD at mid-shaft tibia over the 28 days of HU. However, Dr. Harry Hogan of our research team initiated testing of material properties of the cancellous bone in the proximal tibia over the last year of this project using a novel methodology he has recently developed (see Appendix II). Both the elastic modulus and the ultimate stress of proximal tibia cancellous bone were lower for the hindlimb unloaded group compared to the control animals (**Figure 4,** Appendix I). The difference in means between the groups was not statistically significant, however, due to the rather large scatter in the data. The trends are nevertheless consistent with the decline in trabecular BMD of the proximal tibial metaphysis, which showed a significant decrease in the hindlimb unloaded group. The mechanical properties were also correlated with BMD results to assess the potential predictive value. Both the elastic modulus and the ultimate stress generally increased with higher values of BMD (**Figure 5,** Appendix I). Data from the two different groups of animals are indicated in the figure and show a general trend of lower values for the unloaded group. Results from both groups of animals were pooled together in order to span the broadest range of variables, and correlations were determined for two cases: linear ($y=ax+b$), and power-law ($y=ax^b$). For elastic modulus, the coefficient of determination was 0.39 for the linear equation and 0.51 for the power-law, with neither being statistically significant. For ultimate stress, however, the coefficients of determination were significant for both cases, with a value of 0.61 for the linear ($p=0.013$) and 0.64 for the power-law ($p=0.014$).

Therefore, our data support the hypothesis that HU-induced changes in bone perfusion lead to alterations in bone formation in cortical bone and in BMD and mechanical properties of cancellous bone sites. The loss of BMD, in particular, at cancellous sites implies a focal inbalance between bone formation and bone resorption. We have proposed several mechanisms that could serve to link changes in bone perfusion with skeletal remodeling (see schematic illustration below). The first is the effect that alterations in blood flow may have on interstitial fluid flow and the corresponding production of nitric oxide (NO) and prostaglandin E_2 when exposed to altered flow-induced shear stress. The second is a vascular mechanism through which vascular endothelial cells release substances in response to changes in blood flow and shear stress that may act directly on bone cell populations or serve as paracrine modulators of bone cell activity. Specifically, NO

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and prostacyclin (PGI_2) are potent vasodilators that are, in many tissues, released by the vascular endothelium in response to changes in blood flow, and correspondingly, intravascular shear stress. Thus, this raises the possibility that HU-induced increases and decreases in blood flow and shear stress alter interstitial fluid flow and vascular endothelial cell release of substances capable of modulating bone cell activity, which could subsequently modify the focal balance between osteoblast and osteoclast activity.



Specific Aim II. *To fully characterize the time course of changes in gene expression in bone cells from unloaded and weightbearing bones for modulators of candidate genes involved in mechano-transduction during a 28-d period of hindlimb unloading in the adult rat.*

Allen, M.R., M. Zhang, R.T. Turner, and S.A. Bloomfield. Short-term hindlimb unloading alters TGF-β and cytokine gene expression in femur and humeri of rats (abstract). *J. Aging and Phys. Activity* 8(3): 276-277, 2000.

Dr. Larry Suva's laboratory (SmithKline Beecham Pharmaceuticals) has isolated RNA from humerus and tibia samples from animals suspended for 14 days and from controls. Preliminary analyses with a gene chip array specific to peptides relevant to bone physiology reveal with suspension an up-regulation of integrin abv3 and abv5, nitric oxide synthase, prostaglandin synthase 1 and 2. A down-regulation of several oncogenes (fos, abl) was noted, as well as for BMP receptors types I and II.

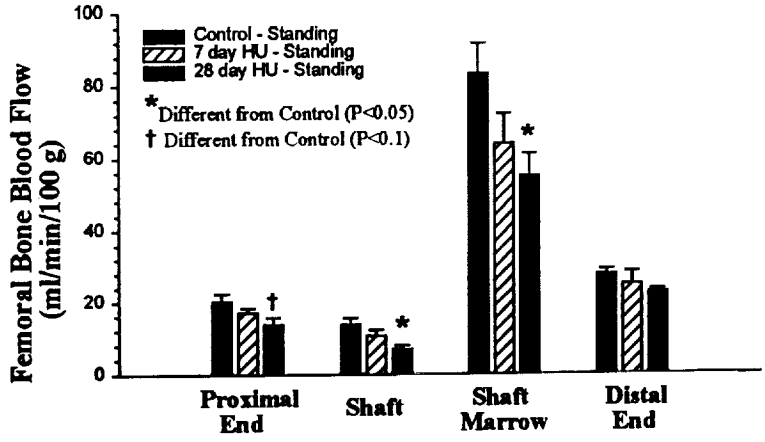
Changes in gene expression have been documented [by Dr. Russ Turner of our research team (Mayo Clinic)] as early as 3 days after the initiation of tail suspension. Although bone matrix proteins (Type I collagen, osteonectin and osteocalcin) have yet to show a change at this early time point, TGF-β_1 is significantly down-regulated and the cytokines interleukin-6 and interferon-γ upregulated in proximal femur samples from 3-d HU rats (**Table 6**, Appendix I). This suggests that peptides important in the regulation of bone cells activity change early in suspension. Notably, however, these changes in expression occur after the rapid decline in blood flow to bone observed after only 10 minutes of tail suspension. By 28 days of HU, larger declines in gene expression are noted for bone matrix proteins, although the decline osteocalcin expression is the only one tested to reach statistical significance.

Utilizing micro-arrays on RNA obtained from ovariectomized rats, Dr. Russ Turner of our research team (Mayo Clinic) has recently detected a membrane kinase previously unidentified in bone cells that is regulated by mechanical loading and estrogen. Northern blot analyses on total RNA isolated from proximal femurs of mature adult rats hindlimb unloaded for 28 days from this project's work reveal a 2-fold increase in expression of this membrane kinase over that detected in cage-activity controls (see **Figure 6, Appendix I**). His laboratory has verified this finding in other RNA samples isolated from rats in previous experiments utilizing either hindlimb suspension or actual spaceflight. This is an exciting finding implicating a membrane protein not previously identified as important in the response of bone to mechanical unloading.

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Specific Aim III. *By administering a β_2 -agonist during hindlimb suspension, to define effects of preventing declines in bone blood flow during unloading on indices of bone mass, geometry, and strength and on gene expression for modulators of nitric oxide and other regulators of mechanotransduction in bone cells. Responses in the unloaded hindlimb bones will be compared with those in the weightbearing humerus and the non-weightbearing calvarium.*

These experiments are currently in progress. The primary reason for the delay in addressing this specific aim was to further investigate the effects of chronic reductions in bone and marrow blood flow on osseous vascular structure. Blood flow was measured during weightbearing activity in 6-month-old control rats (n=10) and in rats following 7 days (n=9) and 28 days (n=8) of hindlimb unloading (unpublished observations). The use of bone blood flow during standing as an estimate of vascular structure is based on the assumption that bone metabolism in control and HU rats is similar when the bones are loaded (body mass was not different among groups), and therefore, differences in perfusion during standing reflect differences in vascular structure rather than metabolic rate. The data from this preliminary study demonstrate that the skeletal blood flow capacity during standing is diminished with 28 days of hindlimb unloading.



Furthermore, these data are consistent with the hypothesis that hindlimb unloading alters the structure of osseous circulation. The data also suggest that skeletal tissue may become ischemic during reloading if vascular alterations result in a reduced bone blood flow capacity that is insufficient to meet the metabolic requirements of steady-state load bearing activity. Such an occurrence in humans following prolonged habitation in microgravity would be deleterious to bone health, and may serve to caution flight surgeons regarding rehabilitation of the skeleton once astronauts return to earth.

APPENDIX I.
TABLES AND GRAPHS OF DATA COLLECTED

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IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

These studies' results impact most directly on potential mechanisms for the bone loss observed with prolonged exposure to microgravity. The dramatic decrease in blood flow to the unloaded hindlimb within 10 minutes of assuming the head-down tail suspension posture implies that this shift in blood flow with altered loading occurs almost immediately upon achieving weightlessness during spaceflight. This effect was maintained or increased by 28 days of unloading, and hence represents a continuing challenge to bone tissue. Declines in whole bone mass occur in those bones experiencing chronic declines in perfusion. Parallel studies on bone structure and mechanical strength demonstrate that these changes in bone mass are likely due primarily to focal changes at cancellous bone sites (e.g., proximal tibia). Given evidence for changes in vascular structure over this time period (28 days), there is little reason to believe that some compensatory change over a longer period of unloading would eventually return blood flow to bone to baseline values. The declines in blood flow to femoral marrow might well impact on osteoprogenitor cell function and account for a decline in mature osteoblast populations commonly observed with real or simulated microgravity; this merits further study.

Whether practical countermeasures that rectify this decline in blood flow to the lower limbs can be developed for use in humans is currently unknown. Countermeasures currently under consideration for bone loss should be evaluated relative to their effects on blood flow to bone. For example, there are encouraging data available from older studies in dogs indicating that long-term physical training can increase bone blood flow even in the resting state (Jurvelin et al., *Acta. Physiol. Scand.*, 1988). However, exercise training as practiced during spaceflight has thus far been relatively unsuccessful in preventing bone loss. It may be that without gravity acting on the skeleton, changes in bone blood flow with exercise are minimal, and that some other countermeasure will have to be combined with exercise. These conjectures are supported by the early studies of Issekutz et al. (*J. Appl. Physiol.*, 1966), which demonstrated that 3 hours of quiet standing per day (but *not* 4 hours of supine exercise) was effective in ameliorating the negative calcium balance seen with prolonged bed rest in healthy young males. Data will soon be available from our intervention studies with a vasodilator pharmacological agent as a "proof of concept"; i.e., does a pharmacologically-induced increase in blood flow prevent some of the decreases in cancellous bone mineral density, bone strength and periosteal bone formation rate in the tibia as observed in our time course studies? If this is the case, then non-pharmacological strategies can be pursued for their effect on blood flow to unweighted bone. These may include lower body negative pressure, passive heating, leg exercise, or some combination of these.

Our data indicate changes in vascular structure after 28 days of hindlimb unloading that result in reduced blood flow even with a brief period of normal weightbearing following unloading. Future studies should follow whether and when compensatory changes occur during recovery from unloading/spaceflight that rectify this deficit in blood flow to bone. These findings have significant implications for the recovery of bone mass following a return to normal gravity; i.e., if changes in vascular structure must occur before blood flow to bone (and presumably bone cell activity) can return to baseline values.

Our data confirm that, in the mature adult rat model, changes in cortical bone mineral density and mechanical strength do not occur over the time period (28 days) used in these experiments. This is in contrast to results documented over the last 20 years in young, rapidly growing rats, which demon-strate significant decrements in bone area, bone mineral density, or both, of unloaded bone. However, there *are* deleterious decreases observed in cancellous bone sites (proximal tibia, femoral neck) in our mature adult rats by 28 days. Although bone researchers may have previously over-estimated decrements in cortical bone sites for mature adults based on young rat data, it is clear that cancellous bone sites are experiencing losses of mineral density and (probably) mechanical strength. These reflect the focal changes noted in humans exposed to spaceflight and bedrest, who experience significant declines in bone mineral density at sites like lumbar spine, femoral neck, and calcaneal bone. Cancellous bone has a greater surface area per unit volume than does cortical bone and is always more vulnerable to alterations in metabolism, so these results are not surprising. Longer duration hindlimb unloading or spaceflight studies are required to confirm whether cortical bone area and strength will ultimately be affected, which seems likely, given that bone formation rate declines by about 90% after 21 days of unloading.

These findings have important implications for earth-bound humans subjected to prolonged bed rest and those restricted, for whatever reason, to using a wheelchair for locomotion. Although the changes in blood flow to bone and to focal remodeling activity may take longer to develop as compared to the strict unloading of weightlessness in spaceflight, over some months of time many of the changes in unloaded lower limb bone are very likely to parallel those observed in these studies. Countermeasures to increase blood flow to bone of the lower limb might then be indicated in these clinical populations.

Finally, gene expression studies utilizing microarrays reveal potentially new candidates for mechanotransduction in bone. These data need to be expanded upon in future studies to better understand the molecular mechanisms involved in bone loss with the chronic unloading of microgravity as well as bed rest.

TABLE 1: ***Body and tissue weight at sacrifice after 14, 21 and 28 days of hindlimb unloading (HU) versus aging controls (CON)***

	0d CON	14d HU	21d HU	28d HU	28d CON
n	7	8	8	7	8
Final Body Weight (g)	433 ± 13	409 ± 16 †	459 ± 13	433 ± 17	456 ± 13
Δ Body Weight (g)	-	-50.9 ± 3.9* †	-32.0 ± 7.1* †	-21.3 ± 9.0	3.5 ± 14
Soleus Wet Weight (mg)	173 ± 11	144 ± 13* †	104 ± 7.7* †	90.6 ± 7.3* †	202 ± 7.1*
Soleus Wet Weight (mg/g BW)	0.398 ± 0.02	0.350 ± 0.03 †	0.226 ± 0.02* †	0.206 ± 0.01* †	0.441 ± 0.22

Values are mean ± S.E. for each group. * indicates significant difference ($p < 0.05$) when compared to 0d CON. † indicates significant difference ($p < 0.05$) from 28d CON

TABLE 2: ***Bone parameters at the proximal metaphysis of tibia and humerus assessed by pQCT after 14, 21 and 28 days of hindlimb unloading (HU) versus aging controls (CON)***

	0d CON	14d HU	21d HU	28d HU	28d CON
Tibia Proximal Metaphysis					
BMC (mg/mm)	12.0 ± 0.43	11.8 ± 0.33	12.0 ± 0.41	11.1 ± 0.37 †	12.6 ± 0.26
Total BMD (mg/cm^3)	670 ± 21	660 ± 17	637 ± 11†	677 ± 12	700 ± 6.0
Total Area (mm^2)	18.2 ± 1.0	18.1 ± 0.60	19.1 ± 0.81	16.5 ± 0.62	18.1 ± 0.43
Cortical Bone Area (mm^2)	8.41 ± 0.25	8.37 ± 0.23	8.32 ± 0.28	7.78 ± 0.22 †	8.71 ± 0.19
Humerus Proximal Metaphysis					
BMC (mg/mm)	7.51 ± 0.08	7.55 ± 0.24	7.39 ± 0.17	7.32 ± 0.27	7.80 ± 0.20
Total BMD (mg/cm^3)	683 ± 16	678 ± 9.0	656 ± 12 †	700 ± 16	700 ± 9.0
Total Area (mm^2)	11.1 ± 0.29	11.3 ± 0.32	11.4 ± 0.37	10.6 ± 0.41	11.3 ± 0.31
Cortical Bone Area (mm^2)	5.43 ± 0.07	5.38 ± 0.18	5.13 ± 0.11	5.14 ± 0.21	5.60 ± 0.17

Values are mean ± S.E. for each group. † indicates significant difference ($p < 0.05$) from 28d CON

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TABLE 3: ***Bone parameters at the diaphysis of tibia and humerus assessed by pQCT after 14, 21 and 28 days of hindlimb unloading (HU) versus aging controls (CON)***

	0d CON	14d HU	21d HU	28d HU	28d CON
Tibia Mid-Diaphysis					
BMC (mg/mm)	7.53 ± 0.12	7.60 ± 0.17	8.47 ± 0.10*	8.35 ± 0.19*	8.32 ± 0.16*
Total area (mm^2)	7.64 ± 0.10	7.62 ± 0.27	8.71 ± 0.25*	8.20 ± 0.22	8.30 ± 0.17*
Bone area (mm^2)	5.47 ± 0.8	5.43 ± 0.12	6.08 ± 0.06* †	5.97 ± 0.13*	5.94 ± 0.11*
Medullary area (mm^2)	2.17 ± 0.06	1.95 ± 0.08	2.63 ± 0.23*	2.24 ± 0.09	2.37 ± 0.08
$CSMI_y$ (mm^4)	5.69 ± 0.35	6.89 ± 1.50	7.09 ± 0.35	6.40 ± 0.34	6.63± 0.34
Humerus Mid-Diaphysis					
BMC (mg/mm)	7.84 ± 0.15	7.90 ± 0.13	8.54 ± 0.20*	8.70 ± 0.07*	8.36 ± 0.17*
Total area (mm^2)	6.13 ± 0.12	6.22 ± 0.13	6.62 ± 0.16*	6.72 ± 0.09*	6.36 ± 0.16
Bone area (mm^2)	5.39 ± 0.10	5.38 ± 0.9	5.84 ± 0.13*	5.90 ± 0.04*	5.64 ± 0.11
Medullary area (mm^2)	0.74 ± 0.03	0.84 ± 0.08	0.79 ± 0.06	0.82 ± 0.07	0.73 ± 0.06
$CSMI_y$ (mm^4)	2.22 ± 0.11	2.57 ± 0.21	2.93 ± 0.29	2.96 ± 0.30	2.68 ± 0.24

Values are mean ± S.E. for each group. * indicates significant difference ($p < 0.05$) when compared to 0d CON.
† indicates significant difference ($p < 0.05$) from 28d CON

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TABLE 4: ***Mechanical properties of tibia and humerus diaphysis after 14, 21 and 28 days of hindlimb unloading (HU) versus aging controls (CON)***

	0d CON	14d HU	21d HU	28d HU	28d CON
Tibia Mid-Diaphysis					
Stiffness (N/mm)	532 ± 16	550 ± 16	588 ± 21	627 ± 45*	577 ± 15
Ultimate Load (N)	238 ± 3	239 ± 8	249 ± 4	246 ± 9	246 ± 8
Elastic Modulus (GPa)	11.6 ± 0.67	11.5 ± 1.10	10.0 ± 0.33	11.9 ± 0.61	10.7 ± 0.32
Ultimate Stress (MPa)	348 ± 16	351 ± 23	342 ± 11	331 ± 15	326 ± 9.1
Fracture Force (N)	180 ± 11	205 ± 10	208 ± 8.5	221 ± 10*	218 ± 9*
Energy to Ultimate Load (mJ)	93 ± 7.5	93 ± 8	123 ± 11	94 ± 9	114 ± 13
Energy from Ultimate to Fracture Load (mJ)	171 ± 21	102 ± 20*	127 ± 11	98 ± 23*	89 ± 24*
Humerus Diaphysis					
Stiffness (N/mm)	362 ± 11	353 ± 12	373 ± 15	386 ± 17	395 ± 13
Ultimate Load (N)	141 ± 5.2	139 ± 4.3	151 ± 4.9	158 ± 5.4*	157 ± 6.4
Elastic Modulus (GPa)	5.93 ± 0.21	5.10 ± 0.27	4.87 ± 0.47	4.96 ± 0.51	5.65 ± 0.40
Ultimate Stress (MPa)	256 ± 6.6	232 ± 6.7	238 ± 13	240 ± 12	252 ± 11
Fracture Force (N)	124 ± 4.2	131 ± 4.0	139 ± 3.2	152 ± 5.9*	146 ± 6.8*
Energy to Ultimate Load (mJ)	49 ± 4.3	66 ± 8.4	61 ± 6.1	66 ± 8.6	62 ± 4.1
Energy from Ultimate to Fracture Load (mJ)	50 ± 10	24 ± 5.2	42 ± 7.4	30 ± 5.1	45 ± 11

Values are means ± S.E. * indicates $p < 0.05$ as compared to 0-d controls.

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TABLE 5. *Histomorphometric measures at the tibio-fibular junction (TFJ) after 7, 14, 21, and 28 d of hindlimb unloading (HU) versus aging controls (CON)*

TFJ	0d CON n=6	7d HU n=8	14d HU n=4	21d HU n=8	28d HU n=8	28d CON n=6
Bone Area (mm^2)	3.023 ± 0.04	3.73 ± 0.20 *	3.25 ± 0.11	3.27 ± 0.08	3.37 ± 0.09	3.34 ± 0.21
Marrow Area (mm^2)	0.833 ± 0.04	1.02 ± 0.08 *+	0.912 ± 0.05	0.794 ± 0.05	0.808 ± 0.05	0.821 ± 0.06
Periosteal MAR (μ/day)	1.585 ± 0.28	1.284 ± 0.16	1.43 ± 0.11	0.628 ± 0.04 * +	0.772 ± 0.02 *+	1.63 ± 0.16
Periosteal BFR (μ/d *100)	131.7 ± 25	70.11 ± 16	97.10 ± 22	13.29 ± 4 * +	17.23 ± 2 *+	103.15 ± 19

* p<0.05 versus 0d CON, + p < 0.05 versus 28d CON

TABLE 7. *Change in gene expression for bone matrix proteins with long term (28-d) unloading: unloaded proximal femur versus cage activity controls.*

Treatment	Control (n=10)	28 Day HU (n=4)	p-Value (t-tests)
Type I Collagen (COL1/18S)	0.255±0.044	0.147±.015	0.16
Osteonectin (OSTN/18S)	0.273±.030	0.185±.026	0.11
Osteocalcin (BGP/18S)	0.378±0.038	0.241±0.018	0.05

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TABLE 6. ***Gene expression for bone matrix proteins, growth factors and cytokines in unweighted femur and weightbearing humerus of 3-day suspended rats versus cage activity controls. Band intensities are normalized to 18S RNA, L32, or GAP.***

Treatment	Control (n=2)	3-day HU (n=3)	p-Value (t-test)
Type I Collagen/18S			
Femur	0.129±0.017	0.093±0.023	0.35
Humerus	0.533±0.235	0.190±0.041	0.16
Osteocalcin/18S			
Femur	1.08±0.180	0.984±0.185	0.70
Humerus	2.85±0.614	1.86±0.194	0.15
Osteonectin/18S			
Femur	0.924±0.135	0.595±0.075	0.10
Humerus	2.24±0.499	1.34±0.100	0.11
IGF-1/L32			
Femur	0.004±1.16E-4	0.004±3.27E-4	0.75
Humerus	0.005±2.38E-5	0.004±0.001	0.23
TGF-β1/GAP			
Femur	1.077±0.054	0.790±0.028	0.01
Humerus	0.532±.053	0.618±0.016	0.15
IL-6/GAP			
Femur	0.024±.002	0.040±.004	0.06
Humerus	0.030±.002	0.032±.002	0.40
IFN-γ/GAP			
Femur	0.013±.0003	0.017±.001	0.07
Humerus	0.017±.0003	0.023±.001	0.02

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Figure 1.
ALTERATIONS IN BONE MINERAL DENSITY OF CORTICAL AND TRABECULAR COMPARTMENTS OF PROXIMAL METAPHYSEAL SITES IN TIBIA (UNLOADED) AND HUMERUS (WEIGHTBEARING)
Tibia (a) and humerus (b) bone mineral density at the proximal metaphysis. Data are presented as mean ± S.E. 0-d (dark bar) and 28-d (light bar) are CON while 14, 21, 28-d (dark bars) are HU animals. * indicates significant difference ($p < 0.05$) when compared to 0d CON. † indicates significant difference ($p < 0.05$) from 28d CON.

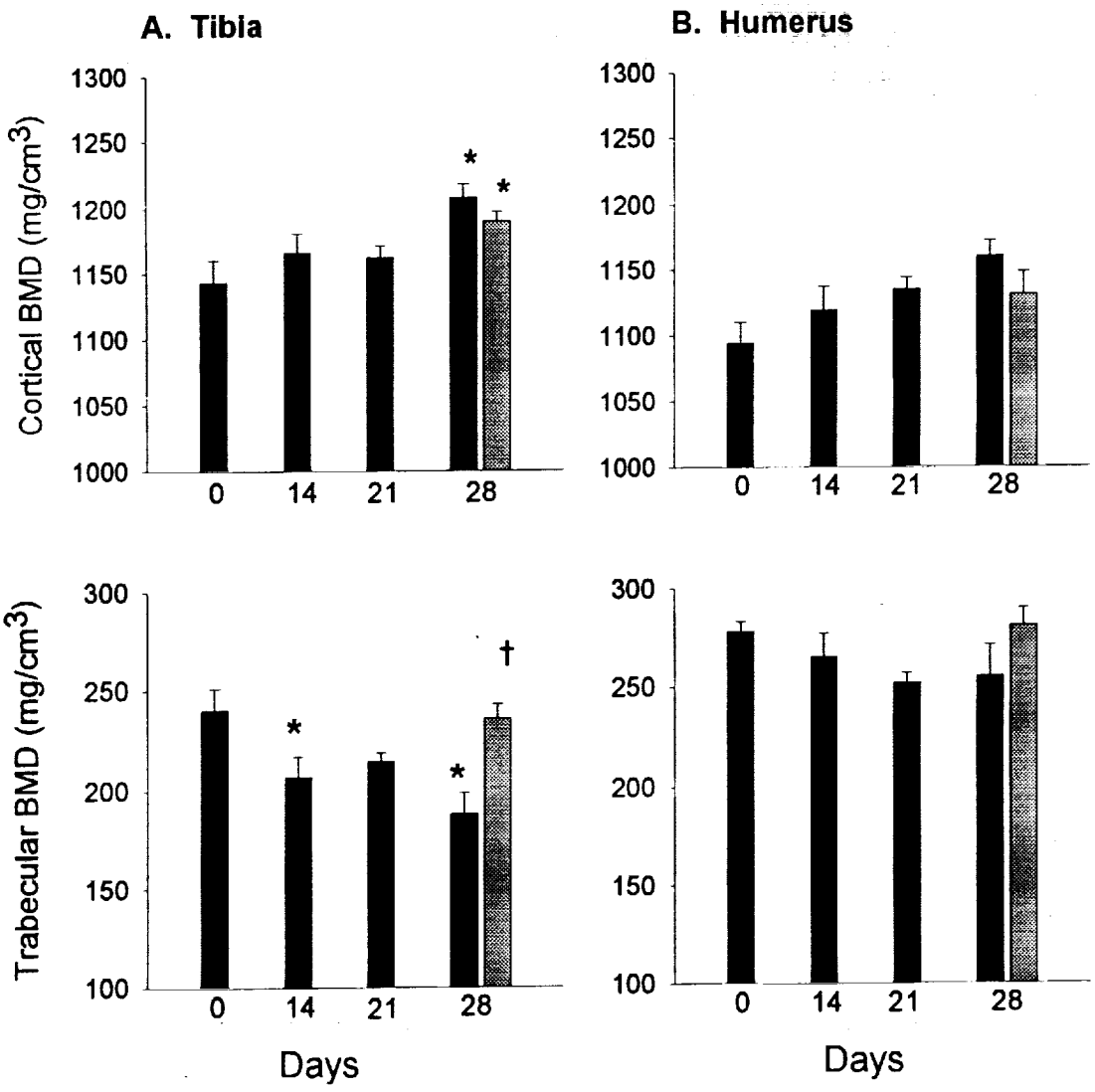


Figure 2.
Alterations in bone mineral density of mid-shaft bone in the tibia (unloaded) and humerus (weightbearing). Tibia (a) and humerus (b) bone mineral density at the diaphysis. Data are presented as mean ± S.E. 0-d (dark bar) and 28-d (light bar) are CON while 14, 21, 28-d (dark basr) are HU animals. * indicates significant difference ($p < 0.05$) when compared to 0d CON.

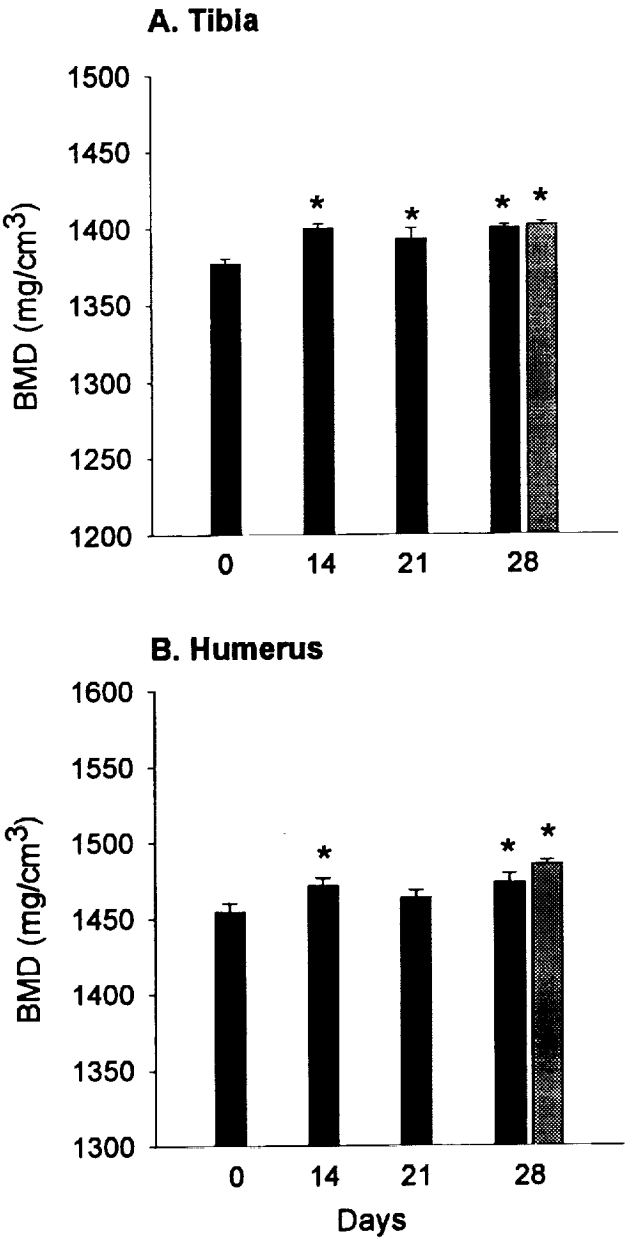
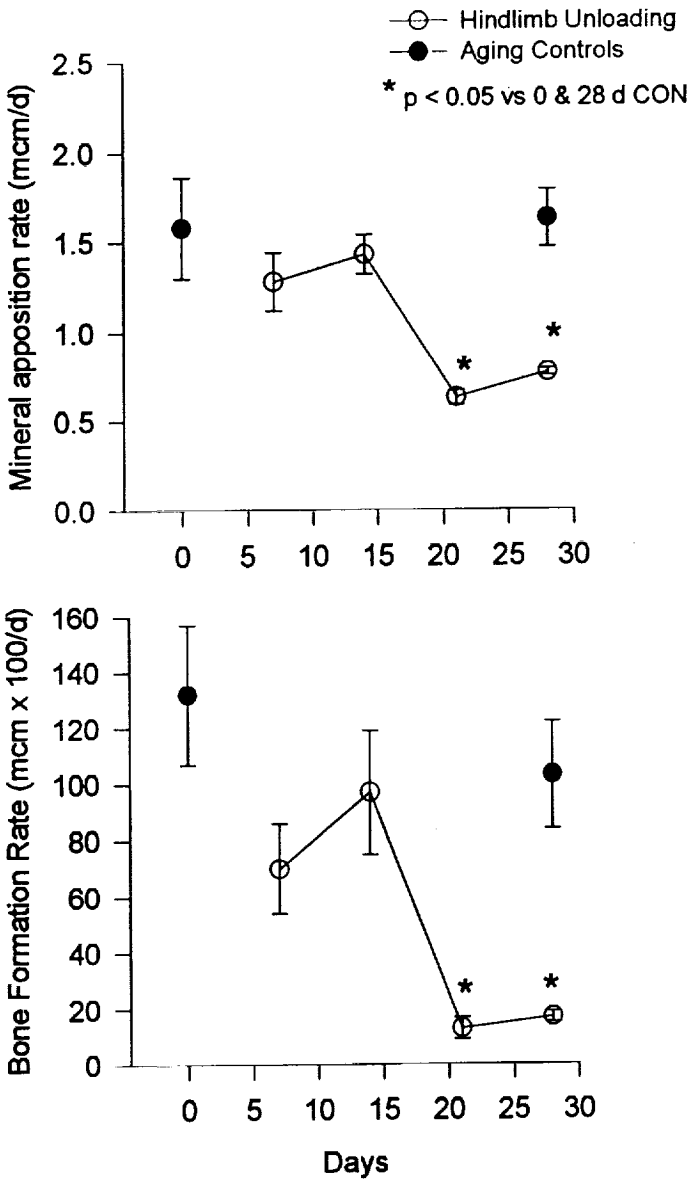
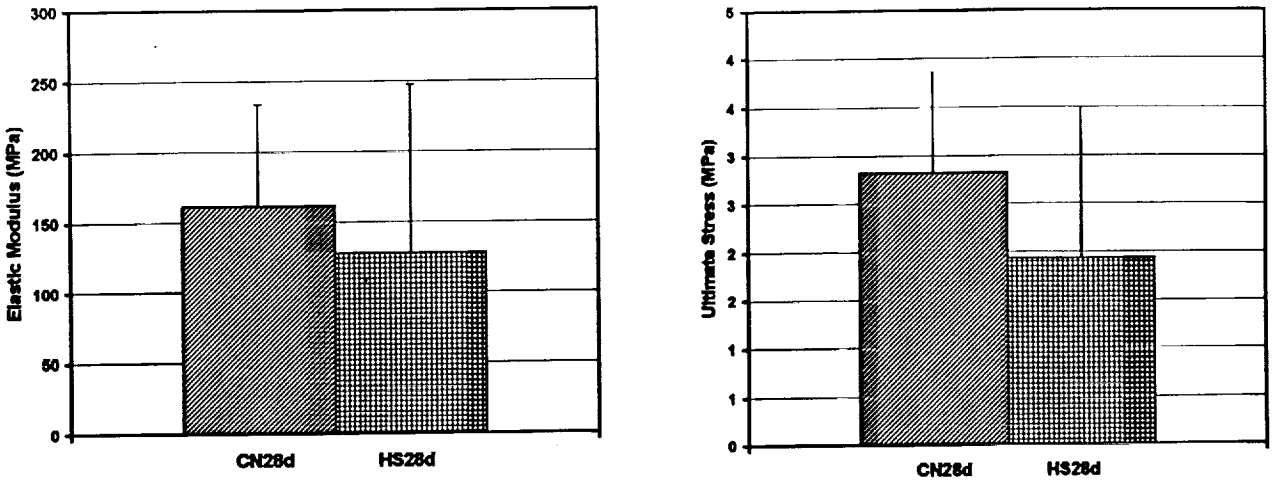


Figure 3.
Time course of declines in mineral apposition rate (MAR) and periosteal bone formation rate at the TFJ in mature adult rats exposed to hindlimb unloading (HU) for 7,14, 21, and 28 days versus aging controls.



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(A) (B)

Figure 4. Proximal tibia cancellous bone mechanical properties by RPC testing (mean +/- SD)

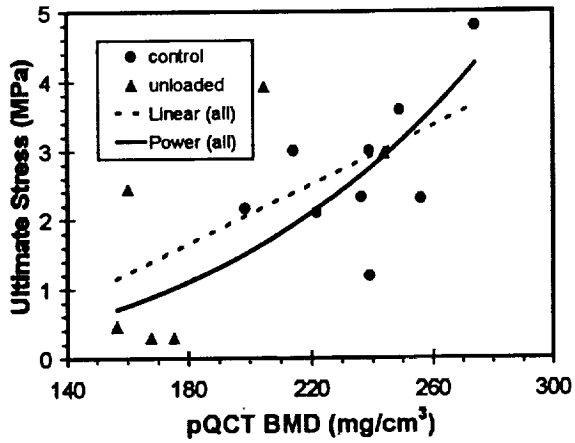
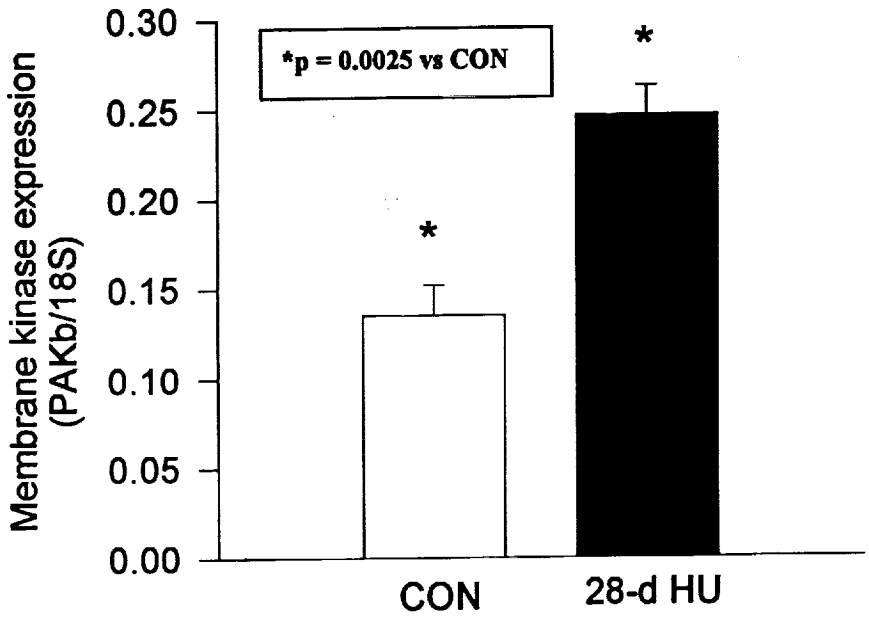


Figure 5. Correlations between proximal tibia cancellous bone ultimate stress and pQCT

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Figure 6. Increased expression of membrane kinase previously undescribed in bone with 28-d HU in mature adult rats.



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APPENDIX II:

NOVEL METHODOLOGY FOR TESTING CANCELLOUS BONE MECHANICAL PROPERTIES—

H.A. HOGAN, PH.D
TEXAS A&M, MECHANICAL ENGINEERING

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NOVEL METHOD OF TESTING CANCELLOUS BONE MECHANICAL PROPERTIES

The method used in this procedure is detailed below. A recent publication details the use of this method at the proximal tibia site in ovariectomized rats:

Hogan, H.A., Ruhmann, S.P., and Sampson, H.W. The mechanical properties of cancellous bone in the proximal tibia of ovariectomized rats. *J. Bone Miner. Res.* 15(2): 284-292, 2000.

METHOD: Mechanical properties were measured for each tibia by reduced-platen compression (RPC) testing. The specimen for this method consists of a slice (nominally 2 mm long) cut from the proximal tibia metaphysis. The specimen thus contains both cortical and cancellous bone and is located distal to the growth plate into the secondary spongiosa. In the current study, the specimen location was further targeted to correspond to the central portion of the region from which pQCT scans were made. High-resolution contact radiographs were made of the proximal half of the tibia to aid in determining the location for each specimen. Images were digitized and analyzed to confirm that the specimen target region was beyond the growth plate and contained secondary spongiosa. Specimens were cut with a low-speed diamond-blade wafering saw with continuous irrigation. With the RPC method, flat, round platens are used to impose compressive loading, but the size of the platens is reduced such that direct contact is made only with the central cancellous bone compartment. The cortical shell remains intact but is not loaded directly. Contact radiographs were made of each cut specimen (axial view) to permit customizing the size of the platens for each specimen. Developed radiographs were digitized and imported into image analysis software. The cancellous compartment was traced manually and the region analyzed to determine the area, major diameter, and minor diameter. The desired platen size was based upon 85% of the minor diameter, but the actual size varied from this somewhat since platens were available in only 0.05 mm increments. Quasi-static loading was applied at a displacement rate of 0.51 mm/min (0.02 in/min) and data recorded at 10Hz. Load-displacement data were reduced to intrinsic material properties, elastic modulus and ultimate stress, assuming uni-axial compression of the cancellous tissue squeezed between the platens. With this idealization, the volume of material comprising the loaded specimen was considered to be the product of the specimen height and the cross-sectional area. It should be emphasized that this approach treats the "material" as macroscopic cancellous bone, which includes both the solid phase trabeculae along with the marrow and void spaces. Intrinsic properties in this case do not refer to the solid trabeculae only but rather represent behavior of the cancellous bone as a whole with differences in specimen size normalized.

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APPENDIX III:

LIST OF ALL PUBLICATIONS SUPPORTED BY THIS NSBRI FUNDING

24

ARTICLES IN PEER-REVIEWED JOURNALS

1. Wilkerson, M.K., J.M. Muller-Delp, P.N. Colleran, and M.D. Delp. Effects of hindlimb unloading on cerebral, splenic, and mesenteric resistance artery morphology. *J. Appl. Physiol.* 87: 2115-2121, 1999.

2. Turner RT. Mechanical signaling in the development of postmenopausal osteoporosis. *Lupus*. 8:388-392, 1999.

3. Delp, M.D., P.N. Colleran, M.K. Wilkerson, M.R. McCurdy and J.M. Muller-Delp. Structural and functional remodeling of skeletal muscle microvasculature is induced by simulated microgravity. *Am. J. Physiol. Heart Circ. Physiol.* 278: H1866-H1873, 2000.

4. Colleran, P.N., M.K. Wilkerson, S.A. Bloomfield, L.J. Suva, R.T. Turner, and M.D. Delp. Alterations in skeletal perfusion with simulated microgravity: A possible mechanism for bone remodeling. *J. Appl. Physiol.* 89: 1046-1054, 2000.

5. McCurdy, M.R., P.N. Colleran, J.M. Muller-Delp, and M.D. Delp. Effects of fiber composition and hindlimb unloading on the vasodilator properties of skeletal muscle arterioles. *J. Appl. Physiol.* 89: 398-405, 2000.

6. Turner, R.T. What do we know about the effects of spaceflight on bone? [Invited review] *J. Appl. Physiol.* 89: 840-847, 2000.

7. Turner, R.T., A. Maran. S. Lotinun, T. Hefferan, G.L. Evans, M. Zhang, and J.D. Sibonga. Animal models for osteoporosis. *Reviews in Endocrine and Metabolic Disorders.* (In press)

MANUSCRIPTS SUBMITTED FOR PUBLICATION

1. Sibonga JD, M Zhang, GL Evans, KC Westerlind, JM Cavolina, E Morey Holton, and RT Turner. Effects of spaceflight and simulated weightlessness on longitudinal bone growth. Submitted to *J Applied Physiology.*

2. Bloomfield SA and Delp MD. Alterations in bone blood flow, bone mass, and bone strength with aging in Fischer-344 rats. Submitted to *Bone* 8/1/00.

MANUSCRIPTS IN PREPARATION

1. Bloomfield, S.A., M.R. Allen, H.A. Hogan, and M.D. Delp. Site-specific alterations of bone mechanical properties with 28 days of hindlimb unloading in the mature adult rat. For submission to *Journal of Applied Physiology.*

PUBLISHED ABSTRACTS

1. Bloomfield, S.A., H.A. Hogan, E.T. Dresser, and J.A. Groves. (1998). Alterations in pQCT-derived bone geometry and volumetric density and in structural properties of femoral neck in senescent rats *Bone* 13 (Suppl 1): S437.

2. Colleran, P.N., J.M. Delp, M.K. Wilkerson, and M.D. Delp. Hindlimb suspension alters arterial morphology in rat hinkdlimb skeletal muscle. *Gravitational and Space Biology Bulletin.* 12(1): 22, 1998.

3. Wilkerson, M.K., J.M. Delp, P.N Colleran, and M.D. Delp. Effects of 14-day hindlimb unloading on rat cerebral, splenic, and mesenteric arterial morphology. *Gravitational and Space Biology Bulletin.* 12(1): 22, 1998.

4. Allen, M.R., C.P. Ingalls, G.L. Warren, and S.A. Bloomfield. (1999). Time course of bone and muscle alterations after hindlimb suspension and 24-hour reambulation in mice. *J. Bone Miner.Res.* 14 (Suppl 1): S217, 1999.

5. Delp, M.D., P.N. Colleran, M.K. Wilkerson, E.P. Lorenz, M.R. Allen, H.A. Hogan, L.J. Suva, and S.A. Bloomfield. Short-term hindlimb unloading diminishes bone mineral density, area and blood flow in rats. *J. Bone Min. Res.* 14:S315, 1999.

6. Demaree, S.R., J.M. Lawler, M.R. Allen, and S.A. Bloomfield. Alterations in antioxidant status in the rat soleus with hindlimb suspension. *Med. Sci. Sport Exerc.* 32(5, Suppl): S104, 2000.

7. Allen, M.R., M. Zhang, R.T. Turner, and S.A. Bloomfield. Short-term hindlimb unloading alters TGF-β and cytokine gene expression in femur and humeri of rats. *J. Aging and Phys. Activity* 8(3): 276-277, 2000.

UNPUBLISHED ABSTRACTS

1. Bloomfield, S.A., M.R. Allen, and H.A. Hogan. Site-specific changes in bone strength and mineral density with 28-d hindimb unloading in the mature adult rat. Presented at the *Integrative Biology of Exercise—Amer. Physiological Society Intersociety Meeting*, 9/22/00.

THE EFFECTS OF PARTIAL MECHANICAL LOADING AND IBANDRONATE ON SKELETAL TISSUES IN THE ADULT RAT HINDQUARTER SUSPENSION MODEL OF MICROGRAVITY.

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1. EXECUTIVE SUMMARY

The loss of bone mass as a result of weightlessness assumes increasing importance as the duration of manned spaceflight increases. To date, efforts to maintain skeletal mass using a variety of exercise techniques or dietary modifications have proven less than effective. **Our central hypothesis was that pharmacological modification of bone resorption combined with precisely designed mechanical loadbearing, may provide the best possible skeletal protection.**

In earlier work, our laboratory rats were placed in a harness and counterweight system that lifted a known fraction of weight off their legs. These data constituted a preliminary dose-response relationship between weightbearing and bone. In our work with NSBRI, we focused on the 50% weightbearing condition to examine the effect of partial weightbearing because it provides a benchmark to estimate the effect of Martian gravity and artificial gravity on bone. Based upon evidence that osteogenesis is particularly sensitive to specific components of dynamic bone strain, we developed a unique instrument to control mechanical loadbearing on the front limbs of hindquarter suspended rats. This consisted of a platform controlled by a digital computer in negative feedback so that it would resonate with specified frequencies of impact. Our system enabled us to process aperiodic forces in the frequency domain as they are applied to bone through normal joint contact and muscle insertions during a form of ambulation. We maintained the normal spectral (Fourier component) composition of quadrupedal forces in a bipedal rat. Partial weightbearing was controlled as a simulation of reduced gravity independently from dynamic forces that simulated carefully designed exercise. We suspected that simultaneous pharmacologic inhibition of resorption and bone turn-over would be more effective with mechanical countermeasures than either regimen alone. We hoped to provide an accelerated model for the NASA JSC human bedrest studies of Lelanc and Shackleford testing the effects of episodic resistive exercise and bisphosphonate therapy because of the more rapid bone turnover in the rat. The endpoints of the proposed treatments will include biochemical, cellular, histological, mechanical, and gross anatomical skeletal analysis in each animal and an assessment of systemic stress. No prior studies have include as completed a range of analytical procedures in the same animal.

1.1 Key Findings

Five experimental conditions were studied in detail. 1. Free roaming rats with full weightbearing on their front limbs (about 30% of total body weight) and unrestrained activity. All free roaming rats were pair fed with hindquarter suspended rats. 2. Free roaming rats pretreated with ibandronate, a potent bisphosphonate. 3. 50% weightbearing hindquarter suspended rats with half of the normal load supported by the front limbs (15% body weight). 4. 50% weightbearing rats, hindquarter suspended as in condition 3, pretreated with ibandronate. 5. 50% weightbearing rats treated on their front limbs with daily episodes of dynamic loading at 3 Hertz as a simulation of ambulatory exercise.

Data is reported on rats that were 3 months old at the beginning of the 35 day trial period and on rats that were 5 months old at the beginning of the 35 day trial period. All were Sprague Dawley female rats housed individually. Data reported as NA is currently being processed.

1.1.1 Temporal changes in bone properties.

The midshaft humeri from all free roaming animals exhibited an increase in polar moment of as a result of changes in cortical geometry, measured by pQCT from the beginning of the study period to the end of the study at day 35.

1.1.2 Comparison between groups in 3 month old animals: Significant changes in individuals from the beginning to the end of the 35 day study period.

Pretreatment of young free roaming rats with ibandronate resulted in significant increases in humeral polar moment, cortical area, sectional modulus and trabecular density.

50% weightbearing rats demonstrated significant reductions in cortical parameters and trabecular density in the humerus.

50% weightbearing rats pretreated with ibandronate exhibited significant increases in all cortical parameters of the humerus: density, area, polar moment of inertia and section modulus.

50% weightbearing rats treated with episodic 3 Hz dynamic loading had significant increases in cortical area, polar moment of inertia and section modulus and trabecular area, but lost trabecular density.

1.1.3 Comparison between 3 month old and 5 month old animals.

In comparison to 3 month old rats, preliminary analysis of older rats exhibited no significant differences in cortical bone properties between the younger and older animals. Preliminary data suggests that older rats are better able to preserve cortical structural properties. Both 3 month old and 5 month old rats at 50% weightbearing show significant decreases in trabecular density.

1.1.4 Post-suspension changes

These measurements included those that could only be performed after sacrifice of the rat including histomorphometry, mechanical testing, bone biochemistry and culture of osteoprogenitor cells. Systemic factors including plasma concentrations of Vitamin D and 24 hour urine production of norepinephrine are also included here. Endpoint pQCT data was compared to the results from other analytical techniques to determine whether the observed differences between treatments could be anticipated from endpoint data alone.

When compared to untreated free roaming animals, 3 month old rats pretreated with ibandronate increased polar moment. 50% weightbearing animals had decrements in trabecular bone density compared with free roaming rats, but polar moment increased and cortical BFR and ultimate stress were maintained. When 50% weightbearing animals were pretreated with ibandronate cortical bone was preserved as indicated by increased polar moment, BFR, ultimate stress, and type I collagen content. Ultimate stress increased more than 50% weightbearing alone. Endpoint pQCT measurements of trabecular bone density decreased. Trabecular density by endpoint pQCT increased. In the group of 50% weightbearing rats treated with supplimentary 3 Hz dynamic loading,

There were no significant differences in plasma vitamin D or urine norepinepherine production compared with free roaming controls.

Table 1: Summary of changes in bone properties in 3 month old female rats

	Free Roaming				Free Roaming + Ibandronate				50% Wtbearing				50% Wtbearing + Ibandronate				50% Wtbearing + 3 Hertz			
	Cortical		Trabec.		Cortical		Trabec.		Cortical		Trabec.		Cortical		Trabec.		Cortical		Trabec.	
PQCT	0	+1	0	-1	0	+1	0	+1	0	-1	0	-1	0	+1	0	-1	0	+1	0	-1
PQCT	0		0		+		0		+		-		+		0		+		0	
Histo.	0		NA		0		NA		0-		NA		0+		NA		0		NA	
Mechan	0				0				0				0				0			
Bioch.	0				NA				0				+				NA			
Cell C.	NA				NA				NA				NA				NA			
Vit. D	0				0				0				0				0			
Stress	0				0				0				0				0			

+ denotes statistically significant increase, - denotes statistically significant decrease, 0+/- denotes no change, pQCT findings are polar moment for cortical bone, density for trabecular bone, top row shows comparison of day 0 to day 35, lower row shows comparsion with Free Roaming group, BFR for cortical histomorphometry , Mechanical testing is represented by ultimate stress, Biochemical analysis of collagen Type 1, Cell culture represented by osteoprogenitor cells, Vit. D. concentration in plasma, Systemic stress represented by 24 hour urine norepinephrine production

1.1.5 Quantification of exercise.

To relate the ground reaction forces to bone strain in our suspended model, we monitored a single axis-strain gauge aligned with the shaft of humerus simultaneously with the ground reaction force on the forepaw. The strain frequency spectrum indicates that less than 1% of the energy comes from components over 10 Hertz. See the examples below (figures 1-2, table 3). Analysis of several hundred paw impacts yielded the following average responses as shown in table 2.

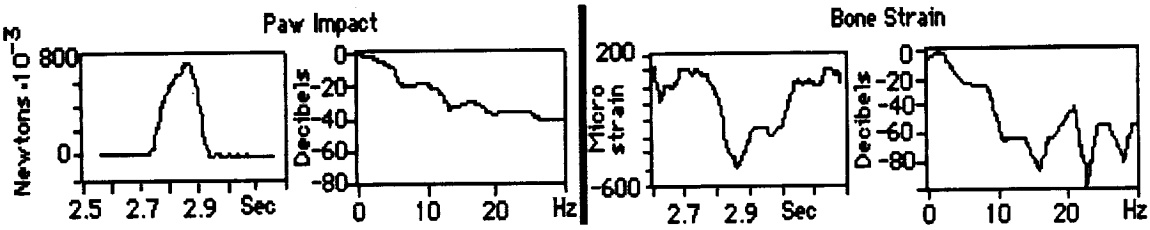


Figure 1: Time and frequency domain representation of paw imapcts and bone strain.

Table 2: Average Limits of Impact Spectrum ± SEM

0 Decibels	-3 Decibels	-20 Decibels
0-1 Hertz	2.1 ± 0.1 Hertz	9.4 ± 0.4

The gain ratio of bone strain/platform accelleration as a log relation of platform vibration frequency (Bode plot) was examined. Ground reaction force is proportional to platform acceleration. A profoundly negative slope of the curves from each animal indicated the low-pass filtering properties of the rat foreleg for oscillations greater than 1 Hertz. High frequency vibrations may be capable of producing bone strain, but are less efficient than low frequency vibrations. More specifically, a ground-reaction force with a 3 Hertz principal component is accentuated by vibration of the floor at the principal frequency. Voluntary muscle activity is well within this frequency and synchronously opposes floor motion.

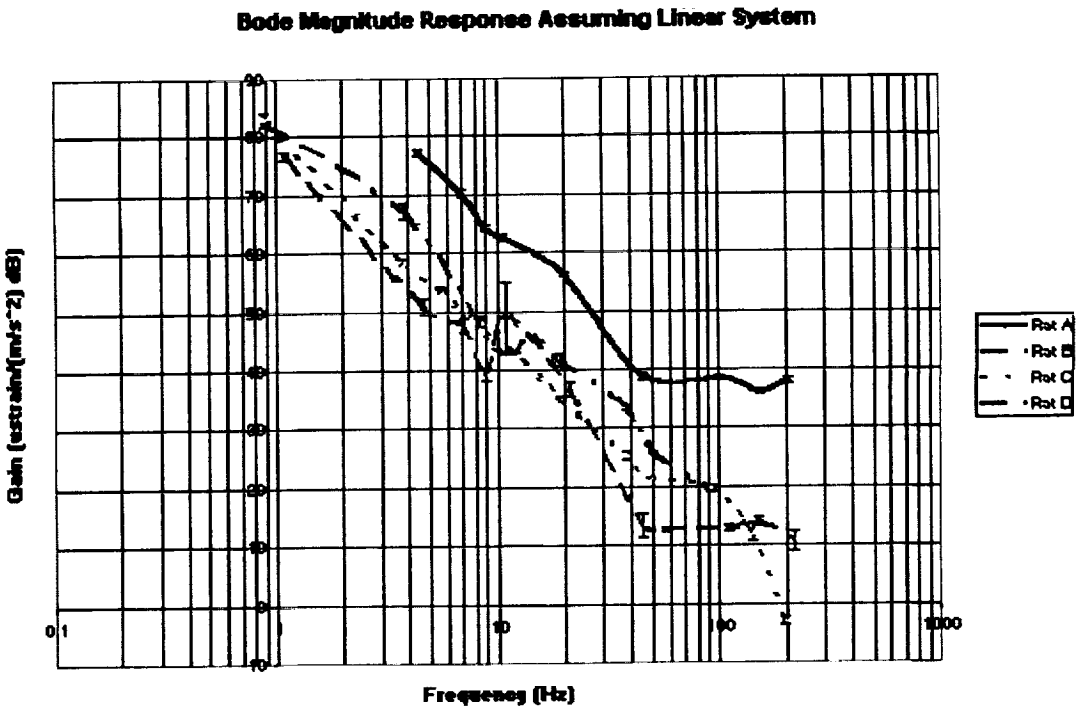


Figure 2: Bode magnitude response of strain/stress versus frequency.

1.2 Summary Of Findings As Related To Specific Aims Of The Original Proposal

1. The hindquarter suspended adult rat model of weightlessness will be studied using a force plate system to apply graded amounts of mechanical loading in order to:

a. Compare the responses of full weightbearing front limb bones to nonweightbearing hind limb bones in partially suspended rats as an indication of bone mass redistribution that may occur in response to regionally specific mechanical loading.

Findings: The project focused primarily on the humerus first because of initial criticisms during site review that the humerus was sufficiently different from the femur that direct comparision between the two bones was not possible. Femurs are still being analyzed.

b. Determine the effect of quantitatively reduced weightbearing on front limb bones in partially suspended rats to simulate appendicular weightbearing at ½ Earth gravity.

Findings: This was accomplished in detail using pQCT, cortical histomorphometry, mechanical testing, bone biochemical analysis and blood vitamin D level measurement. Additional information will be forthcoming as data from cancellous histomorphometry, additional bone biochemistry and cell culture continue to be compiled.

c. Measure the effect of episodic full weightbearing as a model for resistive exercise as a proposed countermeasure on non-weightbearing hind limb bones and on one-half weightbearing front limb bones in partially suspended rats.

Findings: This methodology proved to be faulty in design. The rats exhibited an abnormal posture during periods when they were released from suspension to exercise. The hind legs appeared rather stiff because extension about the knee that persisted throughout the gait cycle. Toward the

end of the 35 day suspension cycle the animals prefered to lie down completely rather than ambulate for each 2 hour period that they were released from their harness.

d. Measure adrenergic receptor density in heart and vasculature as indicators of a generalized systemic "stress" response.

Findings: This technique of assessing systemic stress response was discarded in favor catecholamine measurement in 24 hour urine collections to save time.

2. Define the skeletal actions of ibandronate, a third generation bisphosphonate, as a countermeasure to disuse bone loss in the hindquarter suspended rat model.

a. Evaluate the independent and interactive effects of partial weightbearing, episodic full weight bearing and ibandronate as countermeasures to bone loss.

Findings: The effect of partial weightbearing, partial weightbearing with dynamic loading regimen specifically designed to approximate full weightbearing and the effect of ibandronate was accomplished in detail. Analysis of combination mechanical and ibandronate treatment is ongoing.

3. The specific properties of bone to be compared in control and treated-suspended animals will include: the relative numbers and mineralizing properties of bone marrow stromal fibroblasts, bone mineral mass and density, the biochemical composition of bone matrix , the histomorphometric properties of bone and biomechanical effects of suspension and treatment.

Findings: Bone density, mass, structural analysis was accomplished in detail. Cell culture specimens, cancellous bone and bone chemistry continue to be analyzed. The results will be made available as it is compiled.

1.3 Implications Of These Findings In Reducing Risk For Long-Term Space Missions And Future Research On Earth Medical Problems.

Partial weightbearing at 50% of normal weightbearing appears insufficient to prevent trabecular bone loss, and preserve structural properties of cortical bone. Older animals however seem to be better capable of maintaining important properties of cortical bone. The results of our study suggest that dynamic mechanical loading within the normal ambulatory spectrum is a promising countermeasure. The anisotropic properties of bone are preserved because forces act through muscles and tendons. Trabecular bone density however, were not preserved. Should mechanical regimens fail, pharmacological therapy as a rescue treatment may be helpful. Our results suggest that potent bisphosphonates like ibandronate are helpful in preventing trabecular bone loss under conditions of partial weightbearing. We recognize that even FDA approved agents occasionally manifest untoward effects on organ systems that do not present for years. Crewmembers may opt for medical management of bone loss if diagnostic equipment aboard the spacecraft indicated unacceptable trends of bone loss despite mechanical countermeasures.

We recognize the value of the suspended rat model as a time established simulation for spaceflight. We also appreciate the limitation of rodents as a model for our American astronauts who will be at risk on a mission to the moon or Mars.

2. RESEARCH ACTIVITY

2.1 Methods

In vivo changes in bone density and structural properties at the humerus were evaluated by digital pQCT scans made just prior to suspension and after 35 days of suspension. Rats were anesthetized and positioned in a custom designed frame that held the forelimbs and hindlimbs in a standardized orientation. The pQCT device used was the Stratec-XCT Research SA, Norland Medical Systems. Cortical bone properties were measured at the mid-shaft of the right humerus and trabecular bone properties were measured at the proximal metaphysis of the right humerus.

Mechanical testing by three-point bending was carried out on an Instron 1125 test machine[1] with the bones positioned on two lower supports 12mm apart. The distal section of the diaphysis was loaded by an upper contact pin that contacted the lateral aspect at a location 8mm from the distal end of the lateral epicondyles. This section was selected because it represents the most cylindrically shaped portion of the diaphysis. The displacement rate was 2.54 mm/min and the bones were maintained moist with phosphate-buffered-saline throughout testing. Force and displacement data were recorded digitally using LabTech Notebook Pro software (Version 8.01, Laboratory Technologies Corporation, Wilmington, MA). Force-displacement curves were analyzed with TableCurve (Jandel Scientific, San Raphael CA) to determine the extrinsic whole bone properties. The stiffness (N/mm) was the slope of the linear portion of the curve, the maximum force (N) was simply the greatest value of the load, and the energy absorbed to maximum force (mJ) was the "area" under the curve. Intrinsic material properties were estimated from the relevant structural properties (maximum force [F_{max}] and stiffness [k]) using classical beam theory analysis combined with the pQCT-derived cross-sectional moment of inertia (CSMI) and section modulus (Z) for the axis of bending. The ultimate stress (US) and elastic modulus (E) were calculated by: $US = (F_{max}L)/(8Z)$ and $E = (kL^3)/(48CSMI)$, where L is the lower support span (L=12mm).

Changes in periosteal bone formation rate (BFR) and mineral apposition rate (MAR) were derived from histomorphometric measurements of humeral cross-sections (left side) using Zeiss IIRS microscope equipped with a Zeiss Videoplan 2 digitizing system. The rat bones were double-labeled by intraperitoneal injections of calcein green 10 days and 3 days before of the end of the study.

Biochemical analysis of collagen, osteocalcin and proteoglycans were made from the left humerus. Osteocalcin was measured by commercial IRMA obtained from Nichols Diagnostic Institute, while type I collagen is measured by competitive ELISA methods.

The plasma concentration of 1,25 dihydroxy-vitamin D was determined from retro-orbital blood.

Analysis of catecholamines from 24-hour urine sample collection and measurements of dry adrenal weight were carried out to determine the effects of systemic stress if any introduced by the suspension system.

Five experimental conditions were studied in detail:

[1] Instron Reversible load cell, 5000 lb load cell (100 lb max range), cross-head displacement by LVDT, data acquistion rate of 10 Hz.

1. Free roaming rats that have full weightbearing on their front limbs (about 30% of total body weight) and unrestrained activity. All free roaming rats are pair fed with hindquarter suspended rats.
2. Free roaming rats pre-treated with ibandronate.
3. Partial weightbearing rats that are hindquarter suspended with half of the normal load supported by the front limbs (15% body weight).
4. Partial weightbearing hindquarter suspended rats, with 50% forelimb weightbearing as in condition 3, pretreated with ibandronate.
5. Partial weightbearing hindquarter suspended rats, with 50% forelimb weightbearing, treated on their front limbs with daily episodes of dynamic loading at 3 Hertz as a simulation of ambulatory exercise.

Data is reported on rats that were 3 months old at the beginning of the 35 day trial period and on rats that were 5 months old at the beginning of the 35 day trial period. All were Sprague Dawley female rats housed individually with 12 hour light/dark cycles with lights on at 8.00 am.

Data analysis was reported using mean, +/- SEM with statistical significance ($p < 0.05$) assessed by ANOVA and Student's t test.

2.2 Results

2.2.1 Temporal Changes In Bone Properties Assessed In Vivo On Days 0 And 35 Using pQCT.

This section describes the trends in observed bone properties in each group of suspended and unsuspended (free roaming) rats over a 35-day period. Our focus here is to observe changes in bone properties in different groups of 3 month old (3m) rats over time based on pQCT measurements made in vivo before and after 35 days. Specifically, we attempted to answer the following three questions. First, do cortical and trabecular bone properties change over a 35 day period in 3 month old rats? Second, does partial weightbearing affect the temporal changes in bone properties? Third, how do ibandronate and additional dynamic loading affect temporal changes in bone?

To answer the above questions pQCT measurements were carried out on day 0 and day 35 in different randomly assigned groups of rats. Changes in cortical bone structural properties and trabecular and cortical bone density were assessed. This permitted each rat to be its own control. The changes in bone properties were calculated by considering individual differences in values of bone parameters between day 35 and day 0 for each animal and then averaged for the group. Table 3 summarizes the increases and decreases in pQCT-determined bone parameters as indicated by arrows between pre- and post-suspension values. Statistically significant changes are indicated by "*". Results are shown for free roaming rats (FR), partial weightbearing rats (50%) and rats treated with ibandronate (I) or additional dynamic loading (3Hz).

The results from pQCT measurements of cortical bone made before and after 35 days of suspension showed significant increases in cortical area and polar or 2nd moment of inertia in 3 month old free roaming rats (FR).

Partial weightbearing rats (50%) subjected to 50% of normal weightbearing at the humerus show decreases in all structural properties of cortical bone, significant increases in cortical bone density accompanied by significant decreases in trabecular bone density.

Partial weightbearing suspended animals treated with ibandronate (50%+I) showed significant increases in cortical bone density as well as structural properties of cortical bone unlike untreated partial weightbearing rats. However, ibandronate was unable to produce significant

increases trabecular bone density under partial weightbearing conditions but could increase cortical bone density under such conditions.

Table 3. Temporal changes in bone properties in 3 month old rats.

	Cortical Density	Cortical Area	Polar Moment	Section Modulus	Trabecular Density	Trabecular Area
FR (3m)	⇧	⇧*	⇧*	⇧	⇩	⇧
50% (3m)	⇧*	⇩	⇩	⇩	⇩*	⇩
50%+3Hz (3m)	⇧	⇧*	⇧*	⇧*	⇩*	⇧*
50% + I (3m)	⇧*	⇧*	⇧*	⇧*	⇩	⇩
FR + I (3m)	⇩	⇧*	⇧*	⇧*	⇧*	⇧

Free roaming rats treated with ibandronate showed changes that were similar to control free roaming rats except for changes in cortical density and trabecular density. Unlike control rats and partial weightbearing rats treated with ibandronate, treated free roaming rats showed significant increases in trabecular density.

Dynamically loaded partial weightbearing animals (50%+3Hz) showed changes that most closely mimicked changes in free roaming animals. However, the decreases in trabecular density and increases in trabecular bone area were statistically significant in partial weightbearing rats that received mechanical countermeasures as compared to free roaming rats that showed similar but statistically insignificant changes. Like free roaming animals, animals treated with dynamic loading show significant increases in cortical area and polar moment of inertia as well as non significant increases in cortical bone density.

Thus, it appears that changes in both cortical and trabecular bone properties did occur in 3 month old rats that were subjected to different weightbearing conditions. Partial weightbearing at 50% of normal weightbearing produced changes in bone that were different from those observed at normal weightbearing. Chronic partial weightbearing was ineffective in preserving trabecular bone density. Animals treated with ibandronate or dynamic loading changed the temporal changes in bone as compared to those observed in untreated animals under similar weightbearing conditions. The changes seen in treated-partial weightbearing animals most closely mimicked observed changes in free roaming animals. These results also showed that under conditions of reduced weightbearing and with the application of countermeasures, cortical and trabecular bone responded differently.

2.2.2 A Comparison of Changes in Bone Properties Among Different Groups of 3 month old rats.

In this section, we will take a closer look at changes in bone properties by comparing the differences in observed changes between groups of animals. In particular, we will assess differences between free-roaming animals and different groups of suspended animals and between untreated suspended animals and treated suspended animals. The results presented in this section are targeted towards answering the following questions:

1. Is 50% weightbearing a sufficient countermeasure against bone loss?
2. Do pharmacological and mechanical loading countermeasures prevent bone loss in partial weightbearing animals?
3. Do pharmacological and mechanical loading countermeasures result in changes in bone properties that parallel changes observed in free roaming animals?

This section compares individually normalized percentage changes in bone properties between the different groups of animals focussing on quantitative differences between groups and on evaluating the efficacy of countermeasures. As in the earlier sections, these results are based on in vivo pQCT measurements carried out before and after suspension or 35 days.

The cortical bone density in untreated partial weightbearing rats increased more than in any other group (figure 3). There was no statistically significant difference between groups.

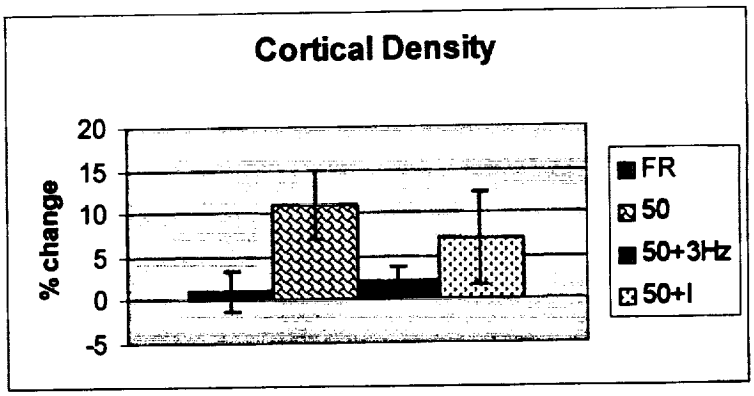


Figure 3: Percentage changes in Cortical Bone Density after 35 days.

The cortical area at the humerus in the partial weightbearing rats that did not receive additional countermeasures decreased significantly as compared to those that received additional countermeasures and as compared to free roaming rats (figure 4). The changes in cortical area in the partial weightbearing rats that received countermeasures are comparable to the changes in free roaming rats.

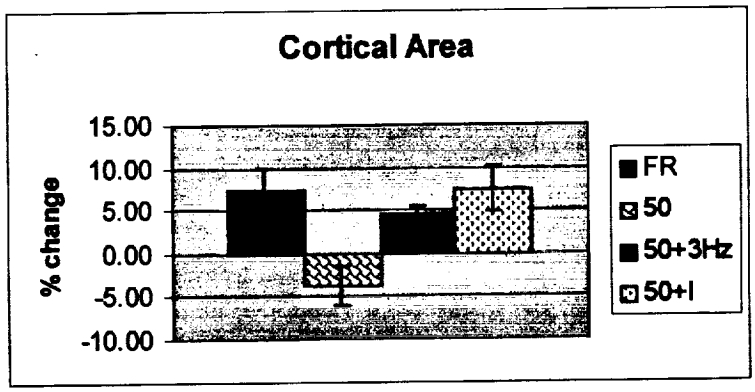


Figure 4: Percentage changes in Cortical Bone Area after 35 days.

The second or polar moment of inertia and section modulus are structural properties that correlate to bending and torsional rigidity and strength respectively. Partial weightbearing rats that did not receive additional countermeasures showed significant decreases in polar moment of inertia as compared to those that received additional countermeasures and free roaming rats (figure 5). The changes in the partial weightbearing rats that received countermeasures were comparable to the changes in free roaming rats.

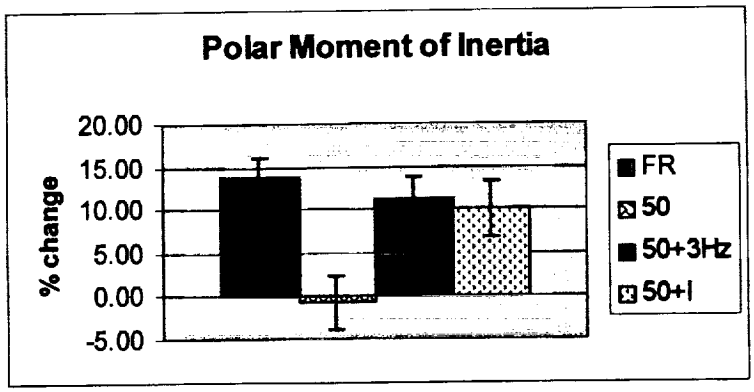


Figure 5: Percentage changes in Polar Moment of Inertia after 35 days.

Unlike the polar moment of inertia, the section modulus in untreated partial weightbearing rats was not significantly less than free roaming rats and rats treated with additional mechanical loading but was significantly less than animals treated with ibandronate (figure 6). There was no significant difference in section modulus in partial weightbearing rats that received countermeasures and free roaming controls.

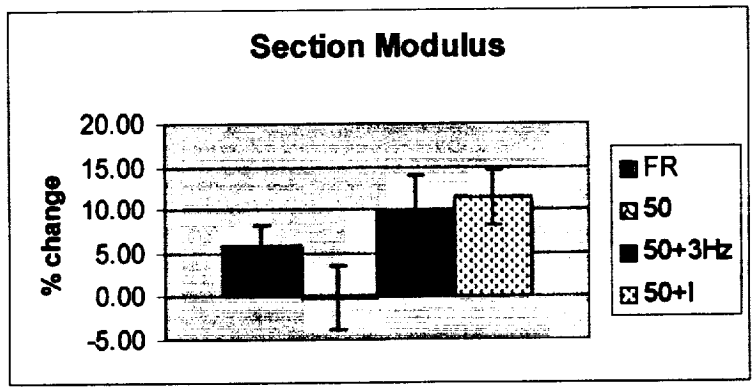


Figure 6: Percentage changes in Section Modulus after 35 days.

Unlike cortical bone parameters, trabecular density decreased in all groups of rats including free roaming rats as shown in figure 7. There was a significant difference in changes in trabecular density between the free roaming rats and untreated partial weightbearing rats and partial weightbearing animals that received additional mechanical loading.

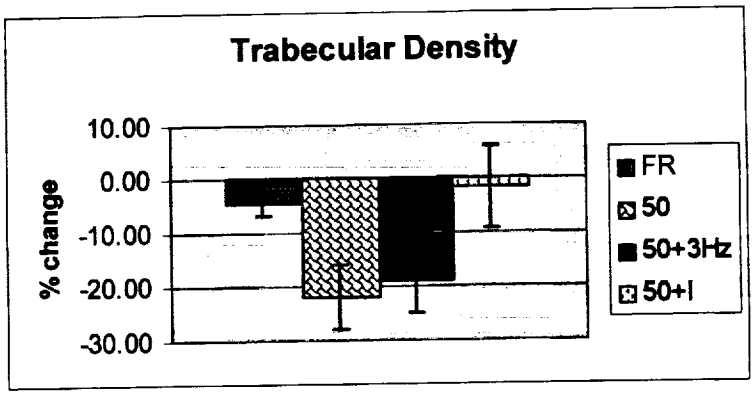


Figure 7: Percentage changes in Trabecular Density after 35 days.

Unlike trabecular density, there was no significant difference in trabecular area between groups (figure 8).

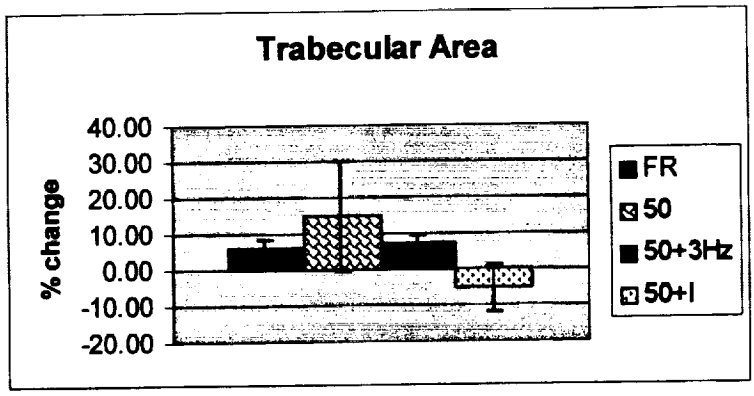


Figure 8: Percentage changes in Trabecular Area after 35 days.

The in vivo pQCT analyses showed that the most significant differences between groups were between 50% and free roaming animals when cortical and trabecular bone properties were considered. There were no significant differences in changes in cortical and trabecular bone properties between free roaming animals and partial weightbearing animals that received countermeasures either as a pharmacological intervention or additional mechanical loading at 3 Hz (except for changes in trabecular density between free roaming and partial weightbearing animals that received dynamic loading). This analysis accounted for individual biological variability amongst the different rats that were randomly assigned to each group. (Since these measurements were made in vivo it was possible to account for differences by treating each animal as its own control, increasing the power of the study).

In summary, partial weightbearing at 50% of normal weightbearing was insufficient to protect against bone loss at the humerus in suspended animals. Both geometrical and structural properties of cortical bone and trabecular bone density decreased. These bone properties seemed to be significantly compromised in untreated partial weightbearing rats as compared to free roaming rats. Pharmacological and mechanical loading countermeasures did preserve some aspects of bone quality under conditions of partial weightbearing. Both ibandronate and dynamic loading at 3 Hz increased the structural properties of cortical bone significantly as compared to untreated partial weightbearing animals and could therefore be considered effective

countermeasures for increasing cortical bone strength. Mechanical loading at 3 Hz however, provided no protection against trabecular bone loss. Ibandronate increased trabecular bone density in free roaming animals but was unable to increase trabecular bone density under conditions of chronic partial weightbearing but was effective in retarding the rate of trabecular bone loss. The goal of pharmacological and mechanical loading countermeasures was not only to prevent cortical and trabecular bone loss but to also ensure that the changes in bone properties were normal; changes should be similar to changes observed in untreated free roaming animals. The results of this study suggested that the observed changes in bone properties in treated partial weightbearing rats were similar to changes in free roaming controls except for changes in trabecular bone density in animals that received additional dynamic loading and changes in trabecular area in animals treated with ibandronate.

2.2.3 A comparison of pQCT changes in 3 month and 5 month old animals.

The main focus of this section is to see the similarity or differences in changes in different groups of 3 month old (3) and 5 month old (5) animals over time based on pQCT measurements made in vivo before and after 35 days. Changes in cortical bone properties expressed as a percent change from baseline in different groups of 3 and 5 month old rats is shown in table 4. Temporal changes are summarized in table 3 where increases and decreases are indicated by appropriate arrows.

Table 4: Percent Changes in Bone Properties measured in vivo on day 0 and day 35 of suspension in 3 month and 5 month old animals.

Group	n	%Change in Cortical Density	% Change in Cortical Area	% Change in Polar Moment of Inertia	% Change in Section Modulus
FR (3m)	8	0.97 ± 0.93	7.47 ± 2.34	13.78 ± 4.33	5.79 ± 2.51
FR (5m)	4	-7.74 ± 2.4	9.83 ± 2.6	14.11 ± 3.14	8.56 ± 5.29
FRI (3m)	7	-0.03 ± 3.24	8.59 ± 3.38	20.44 ± 5.00*	7.86 ± 1.34*
FRI	3	-9.22	-1.952	-45.64	-20.91
50 (3m)	11	11.02 ± 4.00	-3.81 ± 2.35	-0.69 ± 3.05**	-0.04 ± 3.71
50 (5m)	6	-0.27 ± 3.25	-1.70 ± 5.75	11.11 ± 6.23	10.48 ± 4.73
50+I (3m)	6	7.00 ± 5.28	7.45 ± 2.7	10.09 ± 3.19	11.45 ± 3.16
50+I (5m)	3	1.05	3.05	8.65	6.91
50+E (5m)	3	6.01	4.62	5.13	1.66
50+E (3m)	2	3.14	3.58	6.64	4.90
50+3Hz (5m)	2	-4.00	4.06	12.61	9.52
50+3Hz (3m)	7	2.17 ± 1.57	4.56 ± 1.03	11.25 ± 2.54*	9.85 ± 4.07*
50+500Hz (3m)	3	0.31	-7.09	-13.38	-5.60
50+500Hz	1	5.23	4.10	4.63	2.90
50+16Hz (3m)	3	4.00	-0.10	0.85	-0.20

* n = 6
* n = 6
** n = 8
* n = 6
* n = 6

Table 5: Temporal changes in bone properties in older and younger animals.

	Cortical Density	Cortical Area	Polar Moment	Section Modulus	Trabecular Density	Trabecular Area
FR (5m)	⇩	⇧*	⇧*	⇧*	Not available	Not available
FR (3m)	⇧	⇧*	⇧*	⇧	⇩	⇧
50% (5m)	⇩	⇩	⇧	⇧	⇩*	⇩
50% (3m)	⇧*	⇩	⇩	⇩	⇩*	⇩

The results from pQCT measurements of cortical bone made before and after 35 days of suspension showed significant increases in cortical area and polar or 2nd moment of inertia in both 3 and 5 month old free roaming rats (FR). Partial weightbearing untreated suspended rats (50%) at 3 months and 5 months of age show significant decreases in trabecular density.

In addition to comparing temporal changes in each group individually, a comparison of changes in each group was made between 3 and 5 month old rats (figure 9).

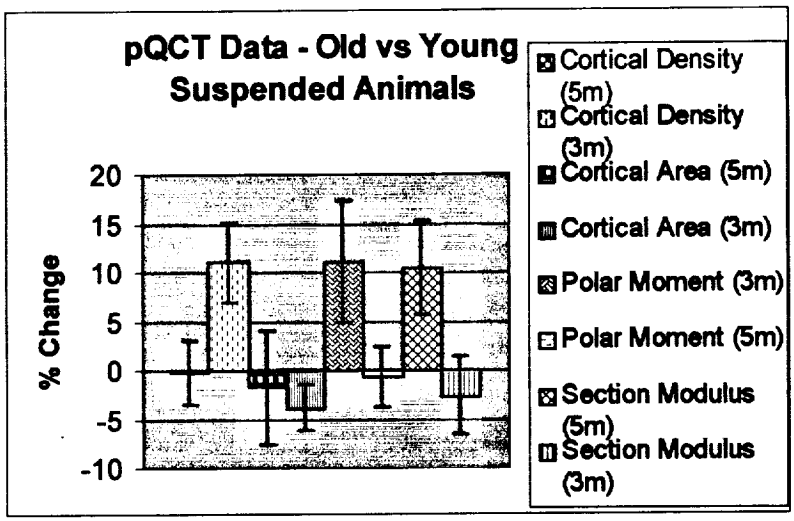


Figure 9: A comparison of changes in bone properties in 3 and 5 month old animals.

Although it appears that older and younger untreated partial weightbearing animals respond differently to chronic reduced weightbearing, the older animals showed increases rather than decreases in bone properties except for cortical bone density although the resultant changes were not statistically different. These results either suggest that the humerus is not affected by growth related phenomena after the age of 3 months, older animals respond less to reduced weightbearing or we may need additional animals in each group.

2.2.4 A Comparison of Bone Properties in Groups of Free Roaming and Suspended Rats: End of Study Data.

This section compares bone properties in the different groups by comparing results that were obtained at the end of the study assuming that all animals had identical bone properties at the beginning of the study based on demographic criteria. The results of this section are important as they can be used to assess whether or not there are differences between the results of longitudinal studies and studies that analyze data based on end of study results.

2.2.4.1 *Results from pQCT*

This section compares bone parameters measured at the end of the study in free roaming rats and different groups of partially suspended rats 3 month old rats. Cortical and trabecular bone properties measured on day 35 are shown in table 6.

Table 6: Cortical and trabecular bone properties measured on day 35.

	Cortical Bone Density g/cc	Cortical Bone Area mm^2	Polar Moment of Inertia mm^3	Section Modulus mm^4	Trabecular Density g/cc	Trabecular Area mm^2
50	1237.09 ± 26.23	4.25 ± 0.15	3.98 ± 0.37	2.65 ± 0.19	346.39 ± 38.17	3.78 ± 0.21
50+3Hz	1231.19 ± 30.55	4.27 ± 0.10	4.19 ± 0.32	2.77 ± 0.11	340.40 ± 40.63	4.17 ± 0.16
50+I	1246.73 ± 25.68	4.46 ± 0.13	4.17 ± 0.17	2.78 ± 0.09	421.17 ± 34.19	4.41 ± 0.41
FR	1277.18 ± 25.68	3.97 ± 0.09	3.22 ± 0.17	2.18 ± 0.08	438.75 ± 15.45	4.45 ± 0.23
FRI	1214.07 ± 39.04	4.49 ± 0.11	4.66 ± 0.26	2.79 ± 0.16	462.15 ± 25.78	4.66 ± 0.47
50+16Hz	1286.967	4.046667	3.44	2.453333	353.1	3.936667
50+500Hz	1258.45	4.00	3.375	2.415	411.4	3.59
50+E	1216.9	3.943333	4.023333	2.543333	299.9	4.5

Figures 10-12 show differences in pQCT data by comparing actual values of each pQCT parameter between groups. There were no significant differences in cortical density between the groups, although free roaming rats (FR) and partial weightbearing rats that received additional dynamic loading at 3 Hz animals had higher cortical bone densities.

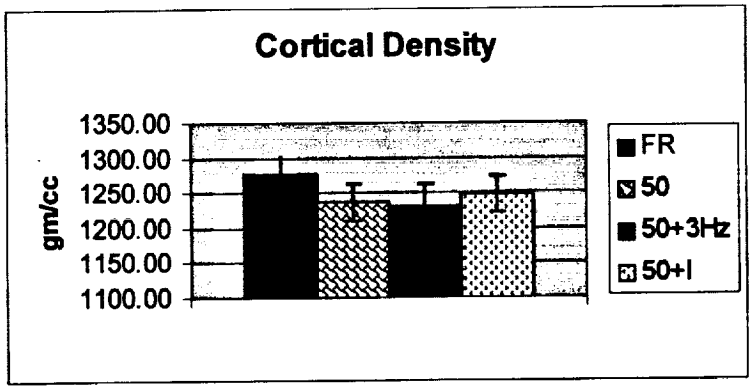


Figure 10: Cortical bone density on day 35.

Unlike the previous analysis where each animal served as its own control, this analysis showed that both cortical bone structural properties (figures 11 and 12) – polar moment of inertia and section modulus were lowest in free roaming animals as compared to any group of partial weightbearing rats. The treated partial weightbearing rats had significantly higher polar moment of inertia as compared free roaming rats (figure 11).

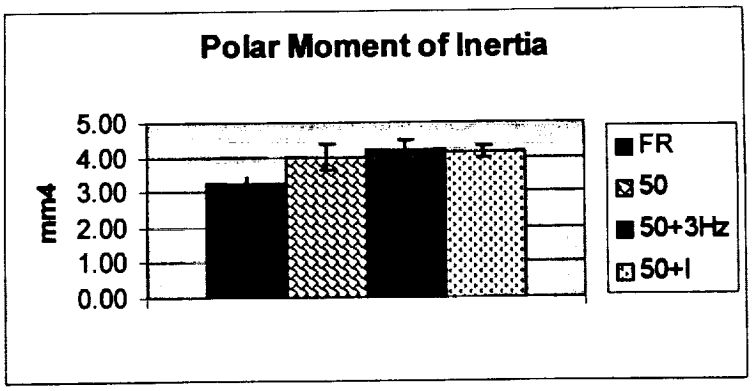


Figure 11: Polar moment of inertia at day 35.

The section modulus was significantly lower in free roaming rats as compared to untreated and treated partial weightbearing rats.

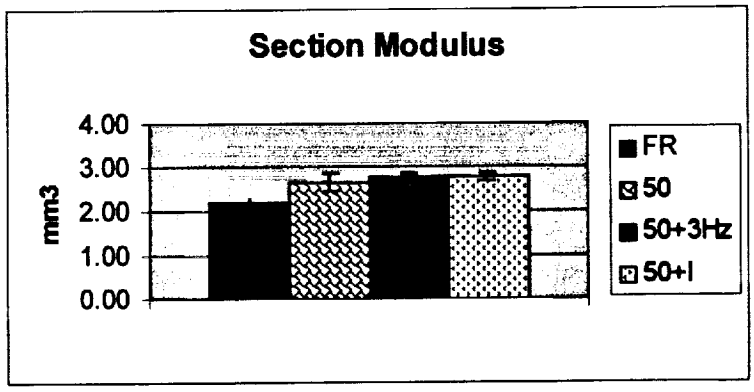


Figure 12: Section Modulus at day 35.

Unlike cortical bone parameters, free roaming rats had a trabecular density that was significantly greater than that of untreated partial weightbearing animals.

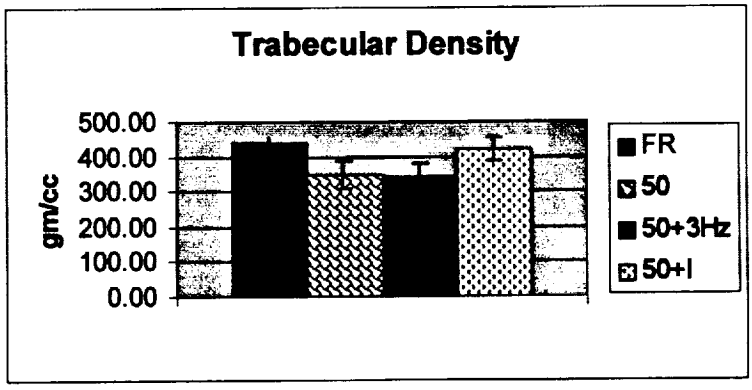


Figure 13: Trabecular Density at day 35.

Thus, pQCT results based on post-suspension data alone suggest that countermeasures applied to partially weightbearing animals were very effective in preserving cortical bone properties

and preventing deterioration of bone structural properties. Ibandronate seemed to be effective in preserving trabecular bone density. Free roaming rats seemed to have the poorest cortical bone properties as indicated by pQCT. Cortical bone structural properties do not appear to be similar in free roaming animals and treated partial weightbearing animals.

2.2.4.2 *Preliminary Results from Mechanical Testing*

The measured extrinisic properties of bone, namely stiffness, maximum force and energy to maximum force showed a greater variation between groups as compared to intrinsic mechanical properties (tables 7, 8). Stiffness was significantly reduced in dynamically loaded partial weightbearing rats as compared to other groups. Ibandronate treated partial weightbearing rats had higher stiffness as compared to other partial weightbearing rats. Although, dynamically loaded partial weightbearing rats showed decreased stiffness, they had significantly higher values for energy to maximum force compared to other groups.

Table 7: Extrinsic mechanical properties of bone.

	n	Stiffness (N/mm)	Maximum Force (MPa)	Energy to Max Force (mJ)
FR	8	392.82 ± 17.81	90.17 ± 1.67	20.08 ± 2.06
FRI	4	347.07 ± 34.22	99.2 ± 1.82	22.07 ± 2.03
50%	3	399.99 ± 26.91	95.20 ± 7.28	17.33 ± 1.27
50% + I	5	517 ± 28.89	110.59 ± 5.34	22.51 ± 1.59
50% + 3 Hz	4	299.72 ± 21.17	109.63 ± 8.33	38.28 ± 4.06
50% + 500Hz	2	338.8	119.78	46.81
50% + E	3	440.3 ± 54.73	99.04 ± 10.87	19.75 ± 1.78

The intrinsic properties, namely elastic modulus and ultimate stress were comparable in all groups except that the elastic modulus, like stiffness in partial weightbearing that received dynamic loading was significantly less than the other groups.

Table 8: Intrinsic mechanical properties of bone.

	n	Ultimate Stress (MPa)	Elastic Modulus (GPa)
FR	8	241.16 ± 18.9	10.610 ± 1.11
FRI	4	200.19 ± 13.93	6.32 ± 0.581
50%	3	225.39 ± 12.00	9.762 ± 0.19
50% + I	5	248.29 ± 16.6	10.901 ± 0.8
50% + E	3	199.57 ± 13.48	9.22 ± 1.81
50% + 3 Hz	4	252.54 ± 8.60	6.639 ± 0.2

2.4.2.3 *Preliminary Results from Histomorphometry*

Histomorphometry results from cortical bone showed that there are no significant differences in periosteal bone formation rates (BFR) among the different groups, except that the rats treated with ibandronate showed significant increases in BFR as compared untreated partial

weightbearing rats (figure 14). Mineral apposition rates (MAR) were comparable for all groups (figure 15).

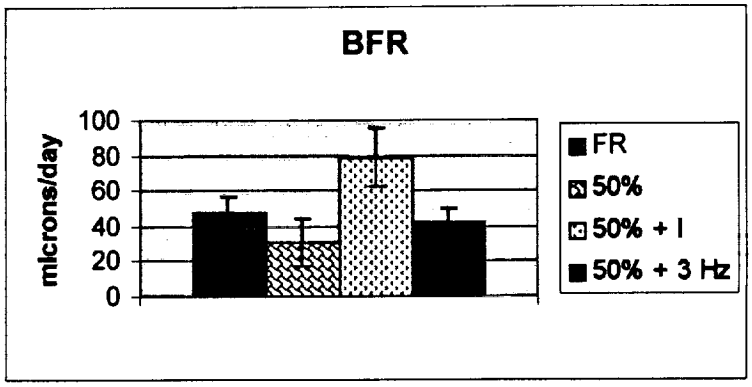


Figure 14: Bone formation rates.

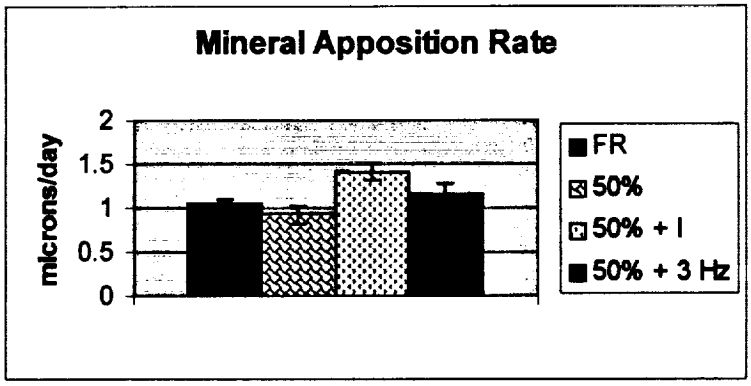


Figure 15: Mineral Apposition Rates

2.2.4.4 *Other Results*

There are no statistically significant differences in norepinephrine levels between suspended and unsuspended rats (1422143 + 557155.9 pg/ml and 723461.6 + 294842.1 pg/ml). Similarly, the adrenal weights for the suspended and unsuspended rats are very similar (0.0277 $\pm$ 0.0019 g and 0.0260 $\pm$ 0.0014 g). Results from biochemical analyses and vitamin D concentration in blood are presented in the Appendix.

2.2.4.5 *Summary of Results*

Based on a comparison of different post-suspension bone properties obtained using both invasive and non-invasive techniques it appeared that ibandronate was the most effective countermeasure for bone loss under conditions of partial weightbearing. Free roaming animals showed lower geometrical properties of cortical bone as compared to suspended animals. It must however, be noted, that an assumption of equal bone properties for each group at the start of the study was made. This assumption however, is incorrect as significant differences in bone parameters between groups exist even after random assignment of rats to treatment groups (see

Appendix). Due to the considerable variability in our data, results obtained by analyzing data from measurement made at day 35 alone should be interpreted with caution.

2.3 Discussion

Growing rats were used in this study as nearly all spaceflight data comes from research on growing rat femurs. Ground-based studies also look at changes in femoral bone properties in growing rats. This study looked at effects of normal and partial weightbearing at the humerus and effects of countermeasures to prevent bone loss in older (5 months) rats. Like other ground-based studies we used the hindlimb suspension model with some modifications but unlike such studies we studied the effects of countermeasures on the humeri which matures earlier than the femur. Our modification of the hindlimb suspension model allowed for quantitative reduction of weightbearing on the forelimbs with the capability of superimposing additional dynamic loading on the ambulatory forelimbs. As stated earlier, our main goal was to evaluate the efficacy of partial weightbearing alone, partial weightbearing superimposed with either dynamic loading or bisphosphonate therapy as countermeasures for bone loss.

Significant changes in bone properties occurred in 3 month old and 5 month old rats, both in response to normal weightbearing and reduced weightbearing. Of importance is the "direction" of changes in bone properties, i.e. whether they increase or decrease bone mass and strength. Under normal weightbearing conditions, three and 5 month old free roaming rats showed increases in cortical area, polar moment of inertia and section modulus indicating increases in torsional and bending rigidity and strength.

The rationale for partial weightbearing at 50% normal was to simulate conditions of Martian gravity since Mars has a gravitational force roughly in that range. Further, it is unknown whether an exploration team to Mars would suffer serious bone loss under reduced gravity conditions or would be spared from bone loss. The results of this study showed that partial weightbearing at 50% normal resulted in significant trabecular bone loss and decrease in structural properties of cortical bone in younger rats. However, cortical bone density is significantly increased. The structural properties of bone were compromised which could be of serious concern if significant bone loss is accompanied by irreversible architectural changes such as disruption of trabecular connectivity or loss of complete trabeculae. Partial weightbearing however seemed to maintain bone formation rate and both extrinsic and intrinsic (material) mechanical properties. Although there were no statistically significant differences between 3 and 5 month old animals, it appears that partial weightbearing maybe sufficient to preserve geometrical properties of cortical bone in older 5 month old animals.

It is known that under chronic reduced weightbearing conditions, bone will adapt its mass (lose bone mass) and structure to the new loading conditions and our results suggest that this occurs even under partial weightbearing conditions. Since dynamic mechanical loading is required to maintain normal bone mass and structure, we proposed additional dynamic loading at 3 Hz in addition to partial weightbearing under conditions of chronic reduced weightbearing to see if the deleterious changes in bone properties could be prevented. Dynamic loading at a frequency of 3 Hz that lies in the normal ambulatory spectrum of the rat may simulate normal mechanical loading as experienced under ambulatory conditions on Earth G.

The results of our study suggest that dynamic loading superimposed on partial gravity conditions was effective in preventing deleterious structural changes in bone. Just as the cortical

area, polar moment of inertia and section modulus (structural properties) significantly increased in growing rats on Earth, similar changes were seen in rats that were dynamically loaded at 3 Hz. Bone formation rate seemed to be maintained under such conditions also. However, trabecular density and the stiffness of bone were significantly compromised under such conditions. Since biochemical analyses were not yet completed in this group of animals, it is not known whether decreased stiffness was due to significant changes in bone matrix composition. Despite decreased stiffness, torsional and bending strength as well as ultimate stress were comparable to free roaming animals. Thus, decreases in material properties were compensated by changes in architecture such as effective distribution of bone mass. The similarity between changes in bone structure and density when compared to free roaming rats suggests that these conditions simulate similar functional demands placed on the skeleton.

Bisphosphonate therapy is used to treat osteoporosis. A powerful bisphosphonate such as ibandronate that can be administered as a single dose parenterally may be effective in preventing bone loss in astronauts for at least three months. This was the rationale to treat partial weightbearing rats with ibandronate. The results of this study showed the beneficial effects of ibandronate in attenuating bone loss. Ibandronate treatment retarded decreases in trabecular density, increased trabecular density under normal weightbearing conditions, increased collagen concentration in bone matrix (results not shown) and bone formation rate. Further, significant increases in structural properties of cortical bone were observed suggesting that bone architecture and strength are conserved by the administration of ibandronate under conditions of partial weightbearing as well as normal weightbearing. Thus, a pharmacological treatment for osteoporosis on Earth, may also be effective under Martian conditions.

An important observation based on pQCT results indicates a large degree of biological variability amongst rats in each group. The results of this study emphasize that longitudinal measurements of bone properties are required to accurately assess changes due to reduced weightbearing conditions and effects of countermeasures. A comparison of pQCT results obtained by comparing pre and post-suspension data and results from end of study pQCT measurements seem to be contradictory. End of study results suggest that under normal weightbearing conditions rats had lower cortical bone strength as compared to rats under conditions of partial weightbearing. This is probably because at the start of the study free roaming animals had significantly lower values for bone parameters as compared to untreated partial weightbearing animals although animals were randomly assigned to groups. However, while assessing longitudinal changes, partial weightbearing conditions without countermeasures results in a deterioration of cortical bone geometrical properties while these properties are enhanced under conditions of normal weightbearing. Thus, results from mechanical testing and histomorphometry must be interpreted with caution. A significantly larger sample size will be required to evaluate the effects of reduced weightbearing and efficacy of countermeasures to account for significant differences in pre-suspension values of bone parameters between different groups if results from the end of the study alone are going to be used.

The stress induced in these animals due to handling and the suspension paradigm and subsequent changes in bone properties if any has not been well characterized. In this study, there were no significant differences in the measured the levels of norepinephrine in urine collected over a 24-hour period in suspended and unsuspended rats. Thus, changes in bone properties could be attributed to growth and chronic reduced weightbearing with or without countermeasures. Weight loss could be considered as an indicator of stress but may also be due to loss of fluid and muscle mass or muscle atrophy.

2.4 Conclusions

The results of this study provide evidence that our central hypothesis "Pharmacological modification of bone resorption combined with precisely designed mechanical loading, may provide the best possible skeletal protection" may be accepted under certain conditions.

The results suggest that 50% partial weightbearing, our simulation of Martian gravity is insufficient to prevent significant trabecular bone loss and deterioration in structural properties of cortical bone but may preserve some aspects of bone quality, particularly in older animals. Pharmacological modification of bone resorption using a powerful third generation bisphosphonate drug, ibandronate coupled with partial weightbearing can attenuate trabecular and cortical bone loss while significantly improving structural properties of cortical bone. Additional mechanical loading at 3 Hz superimposed on partial weightbearing significantly improves structural properties of cortical bone. Both pharmacological intervention and additional mechanical loading change bone structural properties in a manner similar to changes observed unrestrained rats at full weightbearing. The results of this study also underline the importance of assessment of bone quality at different levels of structural hierarchy using different technologies to get a comprehensive overview of changes in bone properties due to unloading and the efficacy of treatment. Most importantly, they underline the importance of in vivo longitudinal measurements of bone properties in the same animal at both cortical and trabecular bone envelopes. Finally, our results suggest that a combination of mechanical and pharmacological countermeasures along with partial weightbearing may yet be effective in the prevention of deleterious changes in bone properties due to unloading as experienced in spaceflight.

2.4.1 Conclusions as related to specific aims of the original proposal

2.4.1.1 *Specific Aim 1:*

The hindquarter suspended adult rat model of weightlessness will be studied using a force plate system for the application of graded amounts of mechanical loading in order to:

a. Compare the responses of full weightbearing front limb bones to nonweightbearing hind limb bones in partially suspended rats as an indication of bone mass redistribution that may occur in response to regionally specific mechanical loading.
b. Determine the effect of quantitatively reduced weightbearing on front limb bones in partially suspended rats to simulate appendicular loading conditions at half-Earth gravity.
c. Measure the effect of episodic full weightbearing as a model for resistive exercise as a proposed countermeasure on non-weightbearing hind limb bones and on one-half weightbearing front limb bones in partially suspended rats.
d. Measure adrenergic receptor density in heart and vasculature as indicators of a generalized systemic "stress" response.

The responses of full or partial weightbearing front limb bones to nonweightbearing hind limb bones in suspended rats was not completed because of initial criticisms during site review that the humerus was sufficiently different from the femur and that a direct comparison between the two bones should not be made. The project focused primarily on the humerus. Data from the femurs is still being analyzed.

The effect of quantitatively reduced weightbearing on front limb bones in partially suspended rats to simulate appendicular loading conditions at half-Earth gravity was assessed using pQCT, histomorphometry, mechanical testing, bone biochemical analysis and blood vitamin D level measurements. The results from pQCT suggest that effect of quantitatively reduced weightbearing on front limb bones in partially suspended rats to simulate appendicular loading conditions at half-Earth gravity is different from the effect of normal full weightbearing and results in deterioration of structural properties of cortical bone. Suspended rats with partial weightbearing on their forelimbs exhibited considerably lower cortical area, polar moment of inertia and section modulus as compared to their free roaming counterparts. Older 5 month old rats showed no significant changes in bone properties except for a significant decrease in trabecular bone density.

Results from mechanical testing and histomorphometry, although less compelling than pQCT data suggest that the intrinsic and extrinsic mechanical properties of bone, bone formation rates and mineral apposition rates in partial weightbearing rats is not significantly different from free roaming animals. Additional information will be forthcoming as data from cancellous histomorphometry, additional bone biochemistry and cell culture continue to be compiled.

The effect of episodic full weightbearing as a model for resistive exercise as a proposed countermeasure on non-weightbearing hind limb bones and on one-half weightbearing front limb bones in partially suspended rats was not completely analyzed as this methodology proved to be faulty in design. The rats exhibited an abnormal posture during periods when they were released from suspension to exercise. The hind legs appeared rather stiff because of extension about the knee that persisted throughout the gait cycle. Toward the end of the 35 day suspension cycle the animals preferred to lie down completely rather than ambulate for each 2 hour period that they were released from their harness.

Specifically designed episodic loading with characteristics of normal ambulation was substituted as an alternative to releasing rats from suspension each day. This work identified the bandpass of a rat footfall during normal weightbearing activity. Virtually all the mechanical energy of a ground reaction impact may be reconstructed from Fourier components with frequencies less than 10 Hertz. More specifically, a ground-reaction force with a 3 Hertz principal component is accentuated by vibration of the floor at the principal frequency. Voluntary muscle activity is well within this frequency and synchronously opposes floor motion.

The technique of assessing systemic stress response by measuring adrenergic receptor density in heart and vasculature as indicators of a generalized systemic "stress" response was discarded in favor catecholamine measurement in 24 hour urine collections because of the time require to obtain and process the data. Our study of urinary catecholamines (presented in earlier progress reports) demonstrated that certain suspended rats exhibited significantly elevated urinary norepinephrine production than control rats while some suspended rats produced no more than their free roaming counterparts. Refinement of our experimental technique eliminated significant differences between control and suspended animals. Possible examination of blood levels of stress hormones from samples obtained at sacrifice by decapitation was also examined as a potential screen for systemic stress. A comparison of hemoglobin and electrolyte concentrations in peripheral blood specimens obtained from retroorbital vessels in vivo to decapitation fluid was performed. This study revealed wide variations in the decapitation fluid and significant differences from peripheral blood. This suggested that stress hormone concentrations obtained from decapitation specimens used in previous studies may be unreliable indicators of systemic stress.

2.4.1.2 *Specific Aim 2:*

Define the skeletal actions of ibandronate, a third generation bisphosphonate, as a countermeasure to disuse bone loss in the hindquarter suspended rat model. Evaluate the independent and interactive effects of partial weightbearing, episodic full weight bearing and ibandronate as countermeasures to bone loss in suspended and control animals.

The effect of ibandronate on bone was studied in the humerus of rats suspended with 50% of their normal weightbearing and in free roaming rats. The effect of ibandronate on young free roaming rats observed from the beginning to the end of the experiment was beneficial and significantly increased structural properties of cortical bone and trabecular bone density. Reduction in trabecular bone observed in 50% weightbearing rats was significantly attenuated by pretreatment with ibandronate. Treatment with ibandronate did not alter expected temporal changes in cortical bone geometry, but it did reduce expected losses in bone density. Section modulus and polar modulus, pQCT parameters associated with bone stiffness and strength were significantly improved over partial weightbearing alone. In 50% weightbearing rats treated with ibandronate, these parameters approximated those found in free roaming animals.

The effects of partial weightbearing combined with additional dynamic loading at 3 Hz resulted in changes in bone properties that closely paralleled corresponding changes in free roaming rats. Although dynamic loading was able to significantly increase structural properties of cortical bone as compared to partial weightbearing alone, it was ineffective in attenuating trabecular bone loss.

The combined effects of partial weightbearing with ibandronate treatment and additional dynamic loading has not yet been evaluated.

2.4.1.3 *Specific Aim 3:*

The specific properties of bone to be compared in control and treated-suspended animals will include: the relative numbers and mineralizing properties of bone marrow stromal fibroblasts, bone mineral mass and density, the biochemical composition of bone matrix, the histomorphometric properties of bone and biomechanical effects of suspension and treatment.

Comprehensive measurements of bone properties using non-invasive and invasive techniques suggest that the effects of partial weightbearing with additional dynamic loading at 3 Hz or antiresorptive treatment with ibandronate as countermeasures to prevent negative effects of microgravity on bone mass are particularly effective on structural properties of cortical bone. Cell culture specimens continue to be analyzed. The results will be made available as it is compiled.

2.5 Appendix

2.5.1 pQCT

Table A.1: pQCT pre and post results from 3 month old rats

Group	Cortical Density Pre	Cortical Density Post	Cortical Area Pre	Cortical Area Pre	Polar Moment of Inertia Pre	Polar Moment of Inertia Post	Section Modulus Pre	Section Modulus Post
FR	1264.81	1277.18	3.70	3.97	2.83	3.22	2.06	2.18
FRI	1217.30	1214.07	4.15	4.49	3.90	4.66	2.59	2.79
50	1123.56	1237.09	4.43	4.25	3.99	3.98	2.65	2.65
50+I	1176.21	1246.73	4.15	4.46	3.78	4.17	2.49	2.78
50+E	1149.30	1216.90	3.82	3.94	3.81	4.02	2.48	2.54
50+3Hz	1205.99	1231.19	4.09	4.27	3.79	4.19	2.55	2.77
50+16HZ	1238.80	1286.97	4.09	4.05	3.51	3.44	2.50	2.45
50+500HZ	1253.90	1258.45	4.29	4.00	3.88	3.38	2.55	2.42

2.5.2 Biochemistry

Biochemical analysis of Type I collagen, proteoglycan and osteocalcin concentrations in the humerus were completed from bone samples removed from 4 treatment groups: free roaming, 50% weightbearing, 50% weightbearing pretreated with ibandronate and 50% weightbearing treated with episodic release from suspension. The rats treated with episodic release from suspension were an early attempt to study the effect of exercise. Later efforts used 3 Hertz dynamic loading and are currently being analyzed. Preliminary data indicates that type I collagen concentration is significantly increased in 50% weightbearing rats pretreated with ibandronate when compared to rats treated with 50% weightbearing alone. Rats at 50% weightbearing alone have significantly lower collagen concentrations than free roaming animals.

The data on collagen and proteoglycan are consistent; both are lost with suspension. The lack of change in osteocalcin indicates that bone loss is occurring in specific sites or pools. It is reasonable that bone loss from partial weightbearing would be accompanied by major bone matrix component loss also. Since the osteocalcein appears to be refractory to hind limb suspension, one might expect the microenvironments of a putative collagen/proteoglycan pool to be diffferent from an osteocalcin pool.

A2: A comparison of biochemical properties.

	Collagen (mcg/mg)	Proteoglycan (mcg/mg)	Osteocalcein (mcg/mg)
Free roaming	100+/-3	100+/-5	99+/-5
50% weightbearing	80+/-9	78+/-11	107+/-14
50% weightbearing + ibandronate	111+/-3	107+/-3	113+/-15
50% weightbearing + episodic release	85+/-3	116+/-8	119+/-17

2.5.3 Systemic Stress

We performed a pilot study of 9 pairs of suspended and nonsuspended control rats for several biomarkers of systemic stress including 24-hour urinary catecholamine production weight gain, dry adrenal mass and adrenergic receptor density (vasculature). Each rat is indexed by a sum of biomarkers ranked by magnitude. The skeleton in a suspended rat with a ranked sum that was significantly different from the mean of nonsuspended rats may have confounding effects of systemic stress with reduced weightbearing. The data from the pilot study depicted below suggested that about 1/3 of the suspended rats had similar levels of systemic stress to the sample population of control animals.

Table A3. The rank sum for individual suspended rats is compared to nonsuspended control rats. Suspended rats #2,3,4 are within 1 SD of the mean of the control group.

Suspended Rat	Sum	Paired Control	Sum
srat 1	40	rat 10	21
srat 2	39	rat 11	21
srat 3	33	rat 12	42
srat 4	38	rat 13	23
srat 5	51	rat 14	33
srat 6	48	rat 15	41
srat 7	40	rat 16	24
srat 8	46	rat 17	37
srat 9	41	rat 18	26
Average	41.77778	Average	29.77778
Std. Dev.	5.562773	Std. Dev.	8.555375

Refinement of our suspension technique eliminated significant differences in urine norepinephrine production between suspended rats and non-suspended or free roaming rats. Comparison of dry adrenal weights from suspended and free roaming rats exhibited no significant differences even in animals with marked differences in urine norepinephrine production. Adrenal weight appeared to be a rather insensitive indicator of systemic stress. There were no significant differences in dry adrenal weight between suspended and free roaming rats.

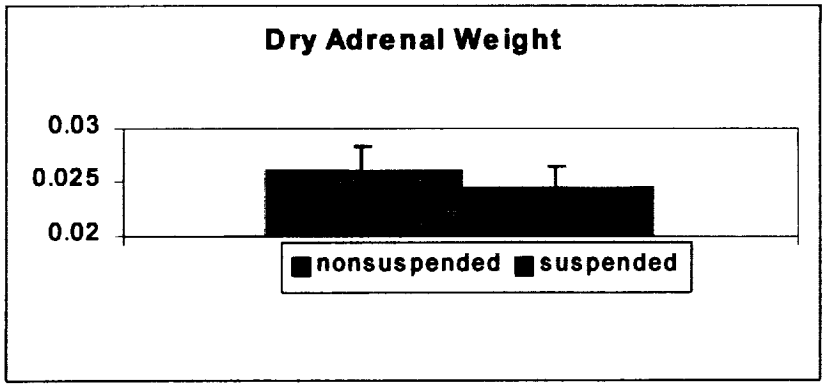
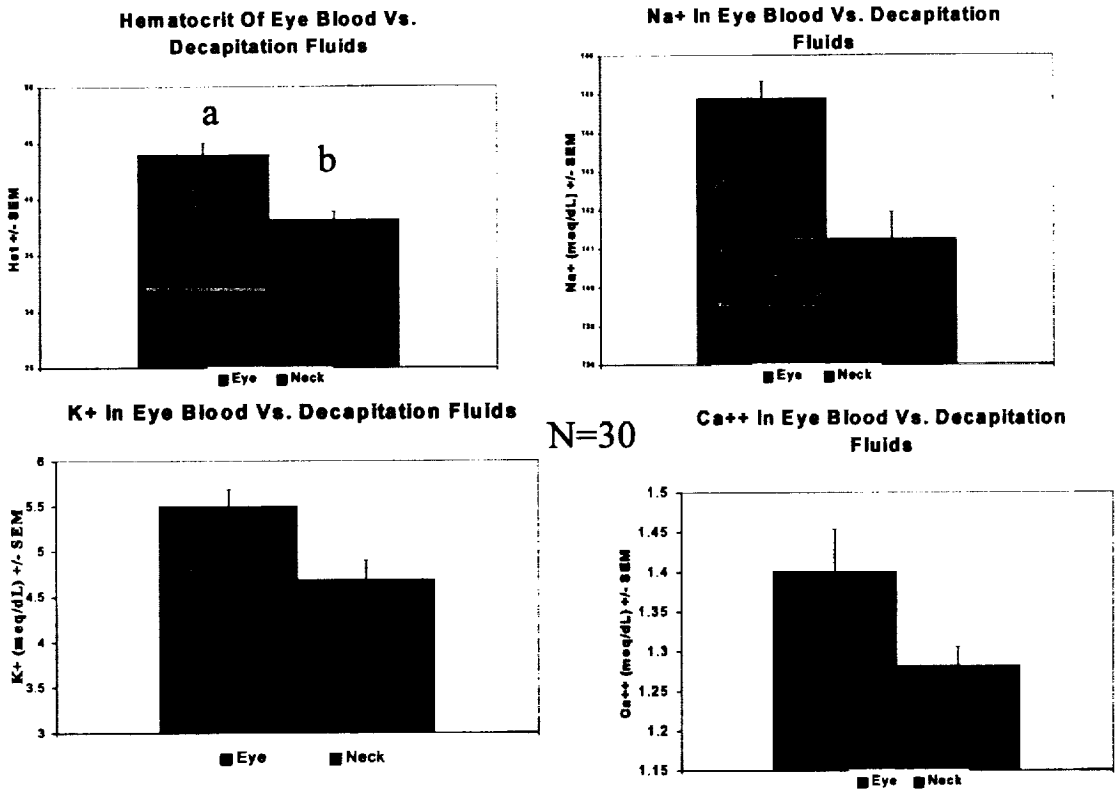


Figure A1: Comparison of dry adrenal weights between free roaming and suspended rats.

Site reviewers suggested that stress hormone levels be studied by collection of blood immediately after decapitation. We compared decapitation blood to a sample from retro-orbital veins using electolytes and hemoglobin concentration as markers. Because our non stress related markers varied markedly between the samples collected from decapitation and retro-orbital veins we reasoned that decapitation fluid did not represent a true blood sample. We further reasoned that collection of sufficient volumes of blood from venous sites might be biased by the stress of venopuncture in an alert rat or anesthesia in sedated animal. Collection of blood samples from an indwelling catheter was not practical in our system.



Stress hormones may not be measured from decapitation fluid.

Figure A2: Analysis of retro-orbital blood in free roaming and suspended rats.

2.5.4 Vitamin D

Blood samples obtained from retro-orbital veins immediately prior to sacrifice were subjected to vitamin D 1-25 analysis. No significant differences were detected between free roaming and suspended rats.

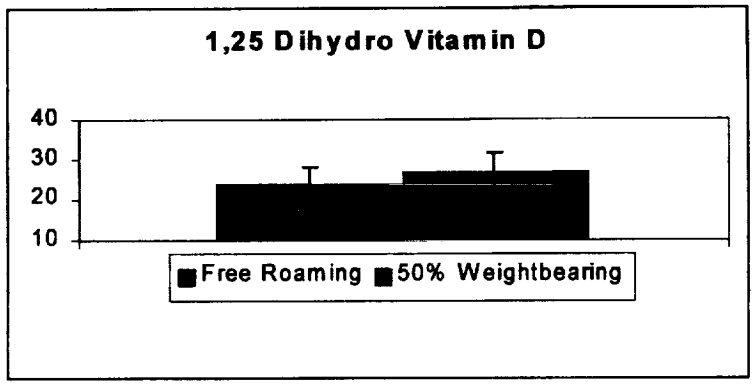


Figure A3: A comparison of levels of vitamin D in free roaming and suspended rats.

3. IMPLICATIONS FOR FUTURE RESEARCH

Partial weightbearing at 50% of normal weightbearing in young, growing 3 month old rats is insufficient to prevent trabecular bone loss, torsional and bending strength as well as structural (skeletal) properties of cortical bone. Bone formation rates, mineral apposition rates and intrinsic properties of cortical bone appear to be maintained. Older animals however seem to be better capable of maintaining torsional and bending strength as well as structural (skeletal) properties of cortical bone. Future studies should investigate the effects of quantitatively titrated levels of weightbearing (0 – 100%) to better understand how bone adapts to different levels of weightbearing and transitions from one weightbearing condition to another. The significance of such studies has implications for both space travel and medical research on Earth. Space travel to the moon or Mars will involve transitions between 1G, conditions of weightlessness and conditions of partial gravity (between 0 – 1G). On Earth, athletes such as gymnasts are subjected to higher weightbearing conditions as compared to elderly, infirm or quadriplegics who subject their weightbearing bones to minimal weightbearing forces and most others have weightbearing conditions between these populations. To design better countermeasures for bone loss in space, such studies would be useful in prescribing countermeasures for bone loss tailored for each astronaut and each space mission. Similarly, in prescribing exercise or pharmacological treatment for osteoporosis it would be helpful to know bone density values as well as levels of physical activity to prescribe optimum treatment.

While we seek to identify a countermeasure for bone loss, it must be acknowledged that the best countermeasure will serve to not only protect the skeleton, but will also benefit other organ systems simultaneously such as skeletal muscle, the heart and the vascular system. As such it emphasizes a better understanding of the potential that artificial gravity may have as the primary countermeasure for each of these systems. Furthermore, if NASA continues its drive toward manned exploration of Mars, data from the reduced weightbearing conditions studied will offer unique insight into the effect protracted exploration of the planet's surface is likely to have on the skeleton.

Exercise as a countermeasure for bone loss has been inconsistently effective in astronauts. This failure may have been a result of noncompliance or ineffective design. Recognition of bone and even an entire limb with its muscular insertions as a mechanical system with maximal rates of movement in normal activity offers insight into the type of "exercise" that is most likely beneficial.

The results of our study suggest that dynamic mechanical loading at 3 Hz in young, growing rats increases torsional and bending strength as well as structural (skeletal) properties of cortical bone under conditions of partial weightbearing. The changes in bone properties observed are quantitatively similar to changes observed under normal weightbearing conditions in free roaming rats. Trabecular bone properties however, were not preserved.

Loading conditions imposed on bone determine its mass, strength and structure including its anisotropic properties. It must be realized that the magnitude and orientation of forces acting on bone act either directly or through the muscles and tendons and changes in muscle properties will also affect forces acting on bone in addition to ground reaction forces. Our preliminary efforts suggest that Fourier analysis of normal activity can direct effective musculoskeletal countermeasures. Dynamic loading can be applied in concert with artificial or partial gravity for synergistic effect on bone and may be considered as a form of muscular exercise. Successful colonization of the moon and Mars will depend on understanding the quantitative relationship

4. LIST OF PUBLICATIONS

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3. Schultheis, L., *et al. Disuse bone loss in hindquarter suspended rats: partial weightbearing, exercise and ibandronate treatment as countermeasures.* in *ASBMR 21st Annual Meeting*. 1999.
4. Schultheis, L., *et al. Partial Weightbearing with a force spectrum typical of normal ambulation improves bone strength in hindquarter suspended rats.* in *46th Annual Meeting, Orthopedic Research Society*. 2000. Orlando, FL.
5. Allen, M., *et al., Ibandronate increases femoral neck bone mineral content, area and ultimate strength in ambulatory hindlimb unloaded rats.* Medicine and Science in Sports and exercise, 2000. **32**(5 (suppl)): p. S270.
6. Daphtary, M., *et al. Effects of Partial Weightbearing, Dyanamic Loading and Ibandronate as Countermeasures to Bone Loss in the Suspended Rat.* in *13 th Humans in Space Conference*. 2000. Santorini, Greece.

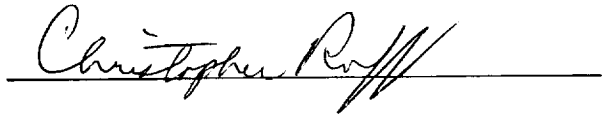
FINAL REPORT, October 2, 2000

NASA NSBRI, Bone Team

Project Title: Skeletal Structural Consequences of Reduced Gravity Environments

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EXECUTIVE SUMMARY

Bone loss under conditions of microgravity has been widely recognized as a potentially critical obstacle to carrying out long-term space missions. Loss of bone mass and density during spaceflight has been documented in both experimental animals and human astronauts and cosmonauts for several decades. However, the structural consequences of such bone loss, in terms of changes in bone strength and increased fracture risk, are much less well understood. Bone strength is a function of both the amount of material present (i.e., mass) and its geometric distribution (i.e., structure). The primary goal of this study was to document changes in mechanically-relevant parameters of bone structure in humans and animal models under conditions of microgravity, and to assess the effects of applied mechanical and pharmacological countermeasures on these changes. Using data derived from a detailed structural analysis of the proximal femur and a dynamic loading model, changes in hip fracture risk after prolonged spaceflight were estimated. The effectiveness of three potential countermeasures – partial weight-bearing, administration of a bisphosphonate, and vibrational mechanical stimuli – on bone strength were evaluated using a tail-suspended rat model. We have thus made major strides towards addressing the goals put forth in the NSBRI Critical Research Path and Bone Team objectives: to assess the probability of a fracture occurence without countermeasures, and to assess the efficacy of different countermeasures in preventing fractures.

The project had three complementary aims : 1) to measure bone structural changes in the hip, extracted from 2-D DXA (dual-energy x-ray absorptiometric) data, in humans subjected to microgravitational conditions, including bedrest on Earth and living aboard the Mir Space Station; 2) to construct 3-D finite element models of the hip from cadaveric femoral specimens which could then be altered using results from (1) to simulate microgravitational effects, and used together with dynamic link analysis to calculate changes in fracture risk; and 3) to assess the effectiveness of several countermeasures - partial (.5 G) mechanical loading, the administration of a bisphosphonate (ibandronate), and mechanical vibrational stimulation - on bone structural changes using a tail-suspended rat model. All of these objectives were accomplished during the three years of support.

Both the bedrest and Mir subjects showed significant declines in measures of bone strength during exposure to microgravity. Declines in the section modulus, an index of bending strength, were comparable in the proximal femoral shaft in bedrest and Mir subjects, averaging almost 1%/month, but rates of decline in the same index in the femoral neck averaged about twice as large in the Mir subjects (1.3%/month) than in the bedrest subjects (.7%/month), despite similar rates of decline in BMD (bone mineral density) (1.3 and 1.15%, respectively). The difference in section modulus changes in the femoral neck was due largely to a small but significant increase in the outer (periosteal) diameter of the bone in the bedrest subjects, an effect not seen in the Mir group. Follow-up studies of the Mir cosmonauts after return to Earth showed an increase in periosteal diameter of the femoral neck which helped to restore its strength, similar to some age changes that we have observed in the normal elderly population. We interpret these findings to indicate that a) geometry, not just bone mineral mass or density, is important in assessing bone strength, b) patterns of change in bone structure in spaceflight subjects are in some ways unique, and thus c) extrapolations from Earth-based studies may be misleading, and

furthermore d) detailed geometric measurements should be included in any bone monitoring protocol during spaceflight and/or Mars exploration.

Two 3-D finite element (FE) models of the proximal femur were constructed from cadaveric specimens from a 36 year-old male and 32 year-old female. 3-D FE analysis allows a much more detailed and realistic modeling of both the geometry and loading conditions of the hip region than is possible using 2-D DXA-derived measurements. The failure loads and risk of fracture following a fall to the side (assuming an average body height and weight) were calculated for each femur, using a dynamic link model that accurately reflects in-vivo loadings. Using the structural information available from the DXA cosmonaut data and some assumptions derived from other experimental studies, these 3-D models were then altered to reflect the average change in structure that would occur after a year of spaceflight, and failure loads and fracture risks were recalculated. Load to failure was reduced by more than 20% on average in the two femora, resulting in an increase in risk for fracture averaging almost 30%. Because we found significant individual variation in how much bone is lost, and how much strength is reduced following exposure to microgravity in both the Mir and bedrest subjects, it is very likely that changes in failure load and fracture risk in some individuals would be even more extreme than these mean estimates. We also compared these results to those obtained through a simpler 2-D curved beam analysis, and found that predicted failure loads were comparable using the two models. The 2-D curved beam analysis has the advantage that it could be applied directly to DXA data gathered during spaceflight, thus enabling longitudinal monitoring of bone changes in astronauts/cosmonauts if a DXA-like apparatus were included on board.

In the tail-suspended rat study, rats were subjected to 35-day periods of partial weight-bearing using a custom-designed platform and mechanical feedback device. Long bone structural parameters were measured before and after treatment using pQCT (peripheral quantitative CT). Results of these studies indicated the following: 1) .5 G loading (similar to the Martian environment) is not sufficient to maintain bone strength. 2) Administration of ibandronate is an effective countermeasure for loss of bone strength under microgravitational conditions. 3) Mechanical stimulation via vibrations applied to the supporting substrate is also an effective countermeasure for maintenance of cortical bone strength, although not trabecular bone density. Thus, a combination of both pharmacologic and mechanical treatments may be necessary to maintain bone strength under microgravitational conditions.

These results show that bone distribution, or structure is a major factor in determining strength and fracture risk. This has implications not only for planning as part of the Critical Research Path for Mars exploration, but also for Earth-based health applications, in particular age-related osteoporosis. Fracture risk is a major medical problem among the elderly. Consideration of all structural components of a skeletal element should improve fracture risk evaluation; in fact, we are currently engaged in parallel studies of bone structural changes in several large demographic samples of the normal population, including the NHANES national survey and the SOF (Study of Osteoporotic Fractures). Studies of these kinds, carried out from a mechanical perspective, should aid in our understanding of both the etiology and consequences of bone loss under a variety of environmental conditions, and provide more accurate evaluation of the efficacy of countermeasures.

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PROJECT RESEARCH ACTIVITY

1) Human Bedrest and Mir DXA Studies

a) Bedrest study

Hip DXA data (Hologic QDR 1000) for six human subjects who had participated in a 112-day bedrest period were provided by Dr. Adrian LeBlanc, our collaborator on this component of the project (LeBlanc et al., 1990). Geometric section properties as well as BMD were derived from DXA data using our custom-designed program (Beck et al., 1990; Mourtada et al., 1996). The program extracts spatial distributional information from individual scan profiles, and integrates this information to derive section properties. A new algorithm was developed for this project that saved bone edge contours and allowed overlay of successive hip scan images during analysis, thus improving longitudinal precision.

A measurement precision study was carried out using multiple scans of the same bedrest patient taken within a 1-3 week period outside of the experimental period (Table 1). Results are

Table 1. Mean, maximum and minimum coefficients of variation in geometric properties from 34 sets of scans on 6 individuals in bedrest study. Each set consisted of 3-4 scans taken over a 1-3 week period.

Parameter		Mean CV%	Maximum	Minimum
Neck Region	BMD	2.0%	0.3%	3.8%
	Cross-sectional area	2.0%	0.4%	3.6%
	Section modulus	2.8%	0.4%	6.1%
	Periosteal diameter	1.1%	0.2%	3.3%
	Endosteal diameter	1.5%	0.3%	4.7%
	Mean cortical thickness	2.2%	0.2%	4.1%
Intertrochanteric Region	BMD	1.4%	0.2%	3.6%
	Cross-sectional area	1.5%	0.2%	3.6%
	Lateral section modulus	2.4%	0.1%	5.2%
	Periosteal diameter	0.7%	0.1%	1.8%
	Endosteal diameter	0.7%	0.1%	1.9%
	Medial cortical thickness	2.1%	0.5%	4.9%
	Lateral cortical thickness	2.9%	0.7%	8.5%
Shaft Region	BMD	1.2%	0.3%	2.7%
	Cross-sectional area	1.3%	0.3%	3.2%
	Section modulus	2.6%	0.5%	8.0%
	Periosteal diameter	0.9%	0.2%	2.6%
	Endosteal diameter	2.6%	0.6%	11.2%
	Mean cortical thickness	1.7%	0.1%	5.1%

shown for three regions – the mid-femoral neck, intertrochanteric region, and the proximal shaft just distal to the lesser trochanter. Average coefficients of variation are less than 3% for all dimensions, indicating acceptable precision. Individual results vary, probably as a function of patient repositioning error, which has a disproportionately large effect on geometric dimensions. This highlights the importance of careful and standardized positioning in hip scanning procedures when data are to be used in structural analyses.

Results of comparisons before and after the 112-day bedrest period in the six subjects are shown in Table 2. Results are expressed as ratios of values after bedrest relative to before bedrest; both the means and ranges for the six subjects are given. Both BMD and geometric section properties are reduced during bedrest. The section modulus, a measure of bending strength, declines 3-4% on average in these subjects, or about 1%/month. The relative decline in BMD in the shaft is equivalent, but is somewhat greater in the femoral neck. The difference in the neck is explained by a small but significant increase in periosteal diameter, which partially counteracts declines in cortical area and cortical thickness. We interpret this increase to be the result of some slight continued mechanical loading of the femoral neck during bedrest, which stimulates periosteal apposition of bone even as overall bone mass is reduced, similar to a pattern we have observed among the elderly normal population (Beck et al., 2000). Individuals show a range of responses to bedrest, e.g., in the femoral neck the change in section modulus ranges from an 8% decline to a 1% gain; thus, loss of femoral neck bending strength can be as much as 2%/month.

Table 2. Measurements at three femur locations normalized to the baseline value and averaged over all 6 bedrest subjects. *Values in parentheses not significant at p = .05, one tailed t-test.

Parameter	Mean Relative Value	Range	Significance*
Neck BMD	0.959	0.933-0.977	.002
Section Modulus	0.974	0.924-1.014	.037
Cortical Area	0.974	0.941-.996	.015
Periosteal diameter	1.015	1.001-1.025	.01
Endosteal diameter	1.028	1.013-1.043	.003
Mean cortical thickness	0.948	0.9240.975	.0006
Intertrochanteric BMD	0.958	0.935-0.988	.003
Section Modulus	0.956	0.932-0.984	.0008
Cortical Area	0.960	0.943-0.994	.002
Periosteal diameter	1.002	0.988-1.018	(ns)
Endosteal diameter	1.009	0.993-1.024	(.065)
Mean cortical thickness	0.988	0.956-1.027	(ns)
Shaft BMD	0.966	0.940-0.985	.002
Section Modulus	0.967	0.941-0.989	.013
Cortical Area	0.970	0.937-1.006	.009
Periosteal diameter	1.004	0.995-1.025	(ns)
Endosteal diameter	1.041	1.017-1.085	.005

Mean cortical thickness	0.952	0.924-0.977	.007

b) Mir cosmonaut study

A total of 84 hip DXA scans were analyzed on 20 Cosmonauts using the same structural analysis program. (This study was carried out in collaboration with Dr. Adrian LeBlanc and his coworkers – see (LeBlanc et al., 1996) for description of original measurements). Data from one subject with only 20 days on Mir were removed; the remaining 19 cosmonauts averaged 178 days on Mir (range 126-312 days). The scan taken closest to launch was then compared to the post-launch scan. There were a total of 26 pre-post flight comparisons on the 19 cosmonauts. Seven cosmonauts had two flights each. Including them as separate comparisons or restricting only to the first flight with data did not change results markedly. Measurements were recorded in three hip regions: 1) at the femoral neck across its narrowest point, 2) across the intertrochanteric region along the bisector of the neck and shaft axes, and 3) across the shaft, 2 cm distal to the midpoint of the lesser trochanter. The average rates of change per month in BMD and structural properties are listed in Table 3. Changes in BMD, endosteal and periosteal diameters, and section modulus are shown graphically in Fig. 1.

Table 3. Mean and range of values for hip BMD and geometric properties before and after spaceflight in Mir cosmonauts.

		Mean %Change/mo	Range	Signif.
Neck	BMD	-1.3%	- 3.8% - 1.7%	<.0001
	Cross-sectional area	-1.2%	-3.6% - 0.2%	<.0001
	Periosteal diameter	0.1%	-2.0% - 2.1%	(ns)
	Section modulus	-1.3%	-4.1% - 0.9%	<.0001
	Mean cortical thickness	-2.0%	-8.5% - 2.7%	<.0001
Intertrochanteric	BMD	-1.4%	-3.6% - 0.2%	<.0001
	Cross-sectional area	-1.4%	-3.6% - 0.5%	<.0001
	Periosteal diameter	0.0%	-0.6% - 0.7%	(ns)
	Section modulus	-1.1%	-4.9% – 1.3%	<.0001
Shaft	BMD	-0.8%	-1.7% - 0.0%	<.0001
	Periosteal diameter	-0.8%	-1.6% - -0.1%	(ns)
	Section modulus	-0.8%	-1.7% - 0.0%	<.0001
	Mean cortical thickness	-0.9%	-1.8% - 0.7%	<.0001

Bone strength, as measured by the section modulus, declines by more than 1%/month in the femoral neck and at a slightly lower rate in the intertrochanteric and shaft regions. Periosteal diameter does not increase at any location. This latter observation is unlike that observed in the bedrest study, or in our studies of the normal elderly population. We hypothesize that the lack of any "compensatory" periosteal expansion in these subjects is due to complete lack of mechanical

stimulation. This hypothesis is also supported by our analysis of post-flight changes in the same subjects (see below).

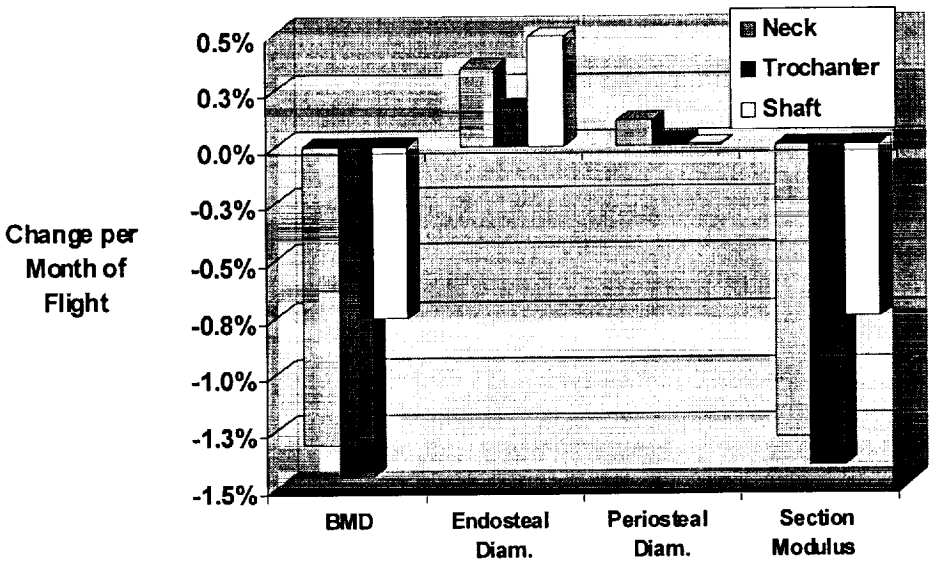


Figure 1. Average rates of change per month in proximal femoral properties in 19 Cosmonauts while aboard Mir.

In addition, follow-up scans at an average of 481 days post-flight (range, 91-894 days) were available for 8 cosmonauts. Fig. 2 shows the results of comparisons between properties just after flight and at the end of this recovery period on Earth for the femoral neck and proximal shaft. Average BMD and section moduli do not return completely to pre-flight levels in either analysis location, but the improvement in section modulus is greater than that in the BMD. Follow-up section moduli are not significantly different from pre-flight values while BMD values remain significantly smaller (marginally in the neck, $p = .07$). The apparent explanation is that after restoration of gravitational loading stimuli, there is a significant increase in subperiosteal width at the neck ($p = 0.028$); an apparent increase at the shaft does not quite reach significance ($p = 0.14$). These data suggest that restoration to gravitational loading produced changes in the femoral neck and shaft that restore section moduli to within 1-1.5% of pre-flight values, moreover this apparent improvement in strength is not readily obvious in the BMD data alone.

These results indicate some interesting parallels and contrasts with age-related osteoporosis that we have observed in the normal adult aging population (Beck et al., 2000). Specifically, declines in bone mass with aging are partially offset by increases in subperiosteal width, which are thought to be stimulated by increased stress due to endosteal (and trabecular) bone loss with aging. During spaceflight, loss of bone mass is not compensated by increases in subperiosteal dimensions (Table 3, Fig. 1), as would be expected in a near-0 G environment. However, in contrast, upon return to Earth and during recovery from spaceflight, subperiosteal breadth does increase. This is again as would be predicted from the normal adult aging model,

i.e., after endosteal bone loss (space flight), exposure to normal gravitational forces (recovery) increases periosteal stresses, which stimulates increase in subperiosteal bone breadth.

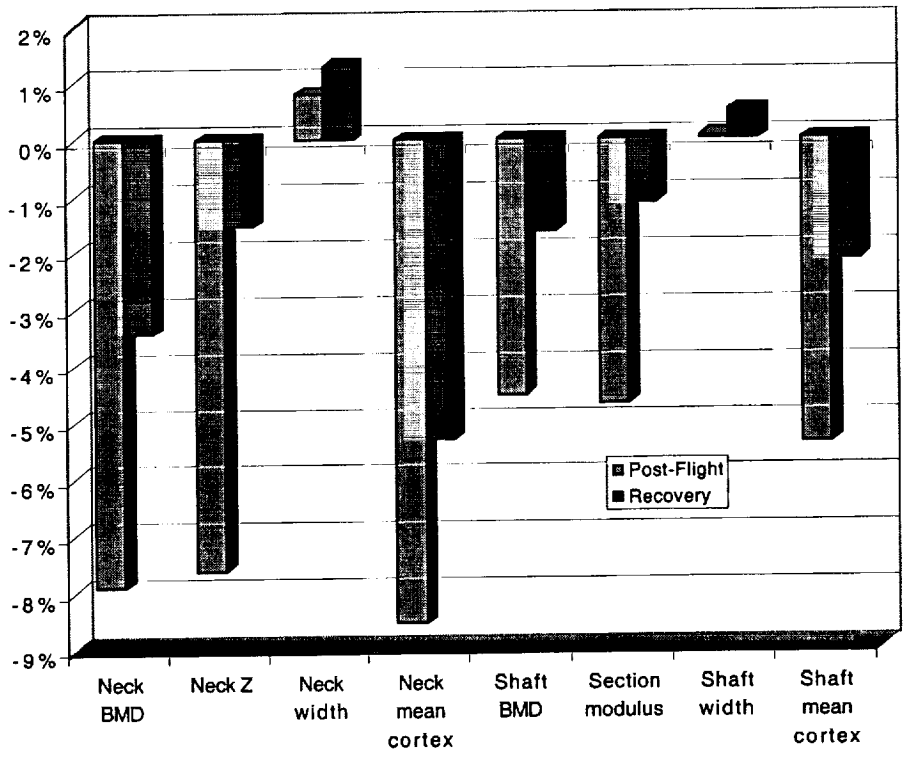


Figure 2. Relative changes in post-flight and follow-up values of BMD, section modulus (Z), subperiosteal width and estimated mean cortical thickness for the femoral neck and shaft in 8 Mir Cosmonauts, compared to pre-flight values (percent change from pre-flight values).

These results also highlight the fallacy of using bulk measures such as BMD alone to predict strength-related changes in bone. In aging there is a consistent discordance between section moduli (strength) and BMD, and we find the same effect during the recovery phase in cosmonauts. The same caveat applies to assessment of potential countermeasures: more direct measures of bone strength are necessary to appropriately evaluate their effectiveness in reducing fracture risk.

2) Finite Element Analyses

Detailed 3-dimensional FE (finite element) analyses were carried out using two cadaveric femora obtained from a 32 year-old female and a 36 year-old male, employing previously described techniques (Oden et al., 1999), modified for this study. Both the geometry and material property distribution for the femur were obtained from high- resolution pQCT scans. Loading configurations of a fall to the side landing on the greater trochanter and mid-stance of gait (with appropriate muscle forces) were applied to the models. The failure loads of the proximal femur in each of these loading conditions were calculated using a custom-written user subroutine in ABAQUS that applied an absolute maximum principal strain failure criterion to the model.

To model the effect of long-term space flight, the thickness of the cortical shell and the density of the cortical and cancellous bone elements of the model were modified to mimic the mean changes in these parameters calculated from the cosmonaut DXA data shown above. Specifically, we first assumed that subperiosteal expansion was absent in microgravity and that bone loss occurred from endosteal and trabecular surfaces only. Based on previous studies (e.g., Kiratli, 1996) a linear reduction in bone mass with flight time did not seem likely, hence we assumed that bone mass was lost at each region at an exponential rate. Rates were derived by first normalizing post-flight cosmonaut cross-sectional area (CSA, i.e., total bone in cross-section) data to the pre-flight value, then fitting the result to a decaying exponential. The resulting half times were 51, 46 and 90 months for the neck, intertrochanteric (IT) and shaft regions, respectively. This fit indicates that bone mass would fall to 50% of the pre-flight value in those intervals.

Fitted rates of change in CSA were then adapted to the (DXA derived) measured geometry for the two cadaver femora so that the CSA declined with flight duration. The model assumed fixed subperiosteal dimensions with circular cross-sections at the neck and shaft, with an elliptical IT cross-section. The anteroposterior dimension of the IT section was assumed to be the measured subperiosteal width at the shaft. Cortical mass fractions of 60 % and 50% were assumed for the neck and IT sections, while the shaft was assumed to be entirely cortical. It was assumed that in the neck and shaft sections, all lost mass was equally distributed between cortical and trabecular compartments.

Cortical porosity cannot be assessed by DXA methods but changes in this parameter can influence failure load magnitudes. Unpublished data from Shaffler (pers. comm., 2000) suggest that cortical porosity increases to about 6% in the unloaded limb, then remains at that level. To account for increased cortical porosity, it was assumed that cortical porosity increased at 6 months to 6%, remaining at this steady-state level for the remainder of exposure to microgravity. Failure was assumed to occur in cortical bone, and the ultimate material properties were assumed to be a function of cortical porosity according to the relationship $E=3.66p^{-0.55}$ described by Shaffler and Burr (1988) (E = elastic modulus, p = porosity).

These parameters were then used to predict changes in medial and lateral cortical thickness at the three section locations for spaceflight durations of 12 months. The predicted dimensions are shown in Table 4 for the male and female femur. These dimensional changes were employed as inputs into the FE models to derive failure predictions.

Two types of comparisons were carried out using the FE models. First, we compared the failure loads (strength) of each long-term space flight model to its corresponding baseline model, to assess change in failure load (maximum load sustained by the hip before failure) as a result of 12 months of spaceflight. Second, we compared factors of risk for fracture, F= Applied Load/Failure Load, before and after 12 months of spaceflight. The applied loads were calculated

using dynamic link models developed as part of this project (Schaffner, 1999), assuming a 50th percentile height and weight male and female.

Table 4. Dimensional changes predicted from space-flight conditions for male and female cadaver femurs.

Female Femur

	Neck			Intertrochanteric			Shaft
Flight months	Medial cortex	Lateral cortex	Trabecular density	Medial cortex	Lateral cortex	Trabecular density	Mean cortex
0	0.249	0.240	0.566	0.630	0.222	0.403	0.699
6	0.235	0.228	0.210	0.578	0.229	0.354	0.652
12	0.209	0.202	0.390	0.508	0.208	0.303	0.560
18	0.185	0.180	0.337	0.449	0.189	0.262	0.485
24	0.165	0.161	0.292	0.397	0.171	0.227	0.424
30	0.147	0.143	0.254	0.352	0.154	0.198	0.372
36	0.131	0.128	0.222	0.312	0.139	0.173	0.328

Male Femur

	Neck			Intertrochanteric			Shaft
Flight months	Medial cortex	Lateral cortex	Trabecular density	Medial cortex	Lateral cortex	Trabecular density	Mean cortex
0	0.145	0.171	0.297	0.407	0.120	0.173	0.391
6	0.138	0.161	0.210	0.376	0.124	0.150	0.371
12	0.124	0.144	0.184	0.335	0.112	0.132	0.329
18	0.111	0.128	0.162	0.298	0.102	0.117	0.292
24	0.099	0.115	0.143	0.266	0.092	0.103	0.259
30	0.089	0.102	0.127	0.237	0.083	0.091	0.231
36	0.079	0.092	0.112	0.211	0.075	0.081	0.206

Results of the FE failure load analyses are shown in Figure 3. The initial predicted failure loads in a fall loading configuration in the male and female proximal femur models were 5086 N and 3610 N, respectively. These failure loads were reduced by more than 20% to 4035 N and 2793 N following the applied cortical thickness, cancellous and cortical bone density changes representing 12 months of spaceflight. The initial factors of risk for fracture in a fall loading configuration were 1.37 (male) and 1.44 (female). After simulated bone changes the factors of risk increased by almost 30 percent.

The initial predicted failure loads in a midstance loading configuration in the male and female proximal femur models were 5992 N and 3912 N, respectively. These failure loads were reduced by over 21% to 4769 N and 3102 N following the applied cortical thickness, cancellous and cortical bone density changes representing 12 months of spaceflight. The initial factors of risk for fracture in a midstance loading configuration were 0.62 (male) and 0.71(female). After simulated bone changes the factors of risk increased by approximately 26 percent. All of these factors of risk assume a 50th percentile height and weight individual.

The overall conclusion from these results is that long-term skeletal unloading as occurs during spaceflight will greatly increase the risk of hip fracture by reducing the strength of the proximal femur. This is true for both a traumatic incident such as a fall and in an activity of

daily living as represented by the mid-stance phase of gait loading. It should also be noted that in this study we only modeled the mean bone loss seen in long-term weightlessness. Due to the wide variation in bone loss observed in the cosmonaut (and bedrest) data, the increased risk of fracture in long-term spaceflight will likely be considerably greater in some individuals, unless effective countermeasures are employed.

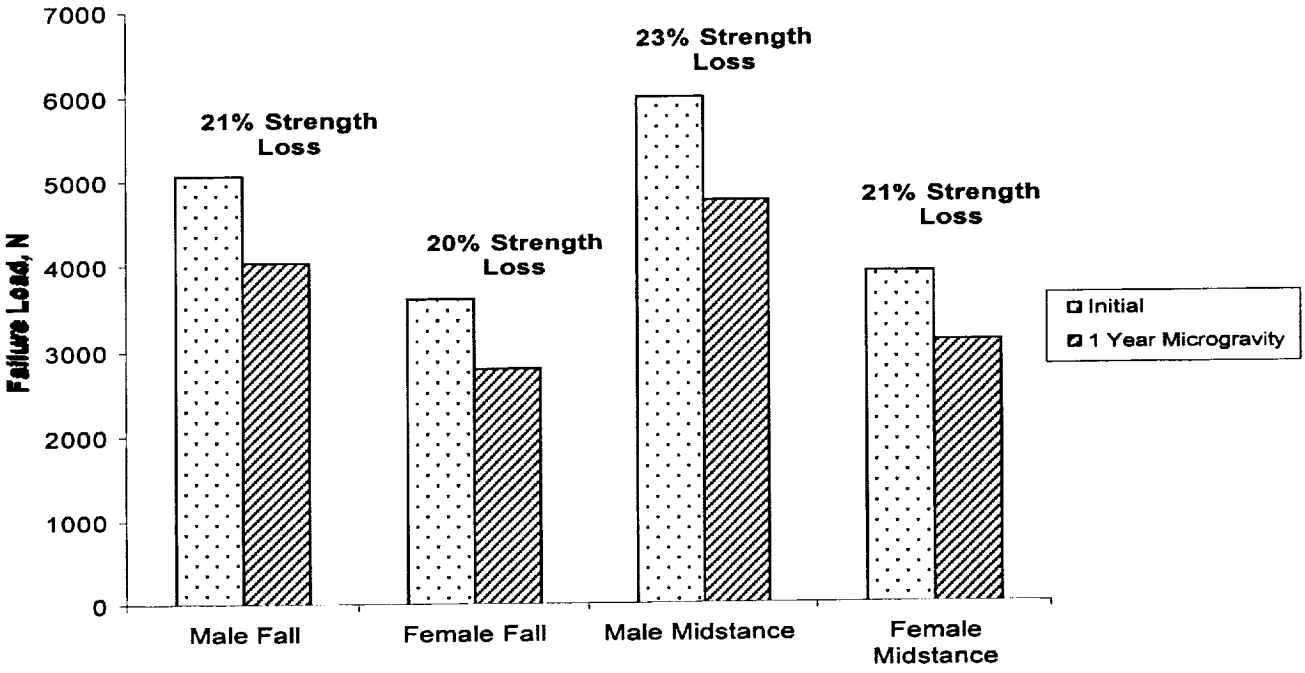


Figure 3. Failure loads for male and female proximal femurs, before and after simulated changes due to long-term spaceflight in both fall and midstance loading configurations.

We also carried out a similar analysis of simulated bone structural changes following 12 months of spaceflight using a simpler 2-D curved beam (CB) model (Mourtada et al., 1996) and the same dimensional changes predicted from the Mir data (Table 4). The approach used in the CB analysis differed from that of the FE analysis in a number of ways. The CB method is not dynamic and strain criteria cannot easily be used for failure definition, hence we used ultimate material properties from the literature for normal human bone, then decremented them to account for increased cortical porosity. The CB model was used to predict changes in limiting strength at the three section locations analyzed by the DXA method for the baseline pre-flight conditions and then for changes expected after 12 months of spaceflight. The goal of the CB analysis was not necessarily to predict load to failure but rather to determine if the decrement in strength to failure was comparable to that obtained with FE analysis.

The loading condition used in the CB analysis was based on a fall on the greater trochanter. This is similar to the fall loading condition employed in the FE analyses, but differs slightly because of the constraints of the 2-D model (i.e., all loadings are constrained to lie in the

frontal plane). Also the load was directed somewhat more inferiorly along the bisector of the neck-shaft angle.

We computed the ratio of pre and post-flight failure strength at the three analysis sections using the following relationship derived from the bending stress equation (ignoring the small contribution of axial load):

$$\frac{F_{post}}{F_{pre}} = \frac{I_{post}}{I_{pre}} * \frac{\sigma_{upost}}{\sigma_{upre}} \qquad [1]$$

where F is the load to failure, I is the cross-sectional moment of inertia, σ_u is the ultimate material stress in tension or compression as appropriate, and the subscripts pre- and post refer to the pre-and post flight condition respectively. Note that the first term on the right in Equation [1] is the effect of changing geometry on stress, which is simplified since the bending moment arm and distance from surface to center of mass is assumed to be constant in this simulation. The second term is the effect of increasing cortical porosity on material failure properties. This term is equivalent to the ratio of pre-and post-flight elastic moduli (E), given by the relationship of Shaffler and Burr (1988) between E and porosity given above. Here we assume that porosity decreases from unity to 0.94 as the cortex get more porous (see above). This provides a second term for the right half of Equation [1] equal to 1.03. Changes in predicted geometry were then used to compute the percent increase in failure strength at the three analysis sites in the male and female cadaver femora. Results are shown in Table 5.

Table 5. Predicted percent change in load to failure from 12 months of microgravity exposure using 2-D curved beam analysis applied to two cadaveric femora.

	Neck	Intertrochanteric	Shaft
Male	-19.6%	-21.9%	-11.0%
Female	-19.1%	-21.3%	-7.5%

The loading conditions for the curved beam model simulation of a fall on the greater trochanter predict failure in the intertrochanteric section (greatest reduction in load to failure), consistent with our observations in-vitro (Beck et al., 1998). The overall magnitude of reduction in failure load is remarkably similar to that obtained using the far more complex FE analyses, where it averaged 21-23% (see above). This suggests that it may be possible to employ the simpler 2-D curved beam model to predict fracture risks where FE analysis is not practical, e.g., in actual monitoring of astronauts/cosmonauts during spaceflight.

3) Tail-suspended Rat Study

Tail-suspended Sprague-Dawley rats were used to investigate the effects of partial weight-bearing and other potential countermeasures on bone loss. A number of different parameters and techniques were used in this study, involving several collaborators. The primary investigator for the study was Dr. Lex Schultheis, and a full report of the study protocol and all study findings can be found in his NSBRI Final Report. The area of responsibility for the

present study was the derivation of bone structural properties in-vivo using pQCT (peripheral quantitative CT), so this report is limited to those findings.

Experimental animals were tail-suspended in such a way that they supported no load on their hindlimbs and about 50% of customary forelimb loads on their forelimbs. The forelimb loading was continuously measured and adjusted using a feedback device coupled with the cage substrate. The 50% load level was chosen for study since it approximates the gravitational field on Mars (.38 G). In addition to the partial weight-bearing, two other primary countermeasures to bone loss were investigated: the administration of a bisphosphonate, ibandronate, and the periodic application of a 3 Hz vibrational stimulus through the substrate (see Schultheis report for details). In initial experiments a third countermeasure – periodic release from the tail suspension – was also tested, but was abandoned later in the study due to problems in "compliance" (rats normally were very inactive during the release period). Initial experiments also measured changes in both the completely unloaded femur and partially loaded humerus, but in later experiments only the humerus was measured. This was done for several reasons: 1) There has been some suggestion that the humerus matures earlier than the femur in this strain of rats (Schultheis, pers. comm.), which could help reduce any confounding effects of rapid growth in the 3-month old animals used in the study. 2) There appeared to be more variation in loading of the femur, since some animals appeared to push off from the sides of the cages with their hindlimbs, i.e., loading varied from 0 G to perhaps significantly greater than 0G in the femur. 3) The pQCT scanning protocol is time-consuming, and scanning both the femur and humerus added considerably to the length of time under anesthesia, contributing to an increased risk of mortality during scanning and loss of experimental animals. 4) Finally, the effects of 0 G on bone have been previously documented, while the effects of partial loading at .5 G have not. Thus, the present report will present results for the larger groups of animals in which the humerus was scanned.

Two locations in the humerus were measured using pQCT: a proximal site, 2 mm distal to the articulation of the humerus with the clavicle, and a mid-distal shaft site, 30% of bone length from the distal end (medial epicondyle) of the humerus. These sites were chosen because they represent areas of primarily trabecular and cortical bone, respectively, and because they can be reproducibly relocated in serial scans of the same animal. Three geometric properties of the shaft site are reported here: cortical area (CA), a measure of axial strength, polar second moment of area (J), a measure of torsional and average bending rigidity, and polar section modulus (ZP), a measure of torsional and average bending strength. One property of the metaphyseal proximal site is reported: trabecular bone density. All of these properties are calculated directly by the software provided with the scanner (Norland/Stratec XCT Research SA).

A total of 45 3-month-old Sprague-Dawley rats were included in this study. (A smaller sample of 5-month-old Sprague-Dawley rats has also been analyzed; since the final animals in this group are still being measured a final report for those additional results will be presented at the end of data collection – see Schultheis Final Report for preliminary comparisons among the older animals.) The animals were divided into five approximately equally sized groups according to the following treatments: Group 1: free-ranging controls; Group 2: 50% weight-bearing; Group 3: 50% weight-bearing + administration of ibandronate; Group 4: 50% weight-bearing + periodic mechanical stimulation; Group 5: free-ranging + administration of ibandronate. The total experimental period was 35 days, with pQCT scans taken on the first and last days.

3-way analyses of variance (ANOVA) were carried out with the percent changes in bone structural parameters over the experimental period as dependent variables and 50% weight-bearing, ibandronate, and mechanical stimulation as factors. Results of these analyses are shown in Table 6. In addition, the differences in percent changes between the control group and individual experimental treatment groups were evaluated using t tests, and are shown graphically in Figure 4.

Table 6. Effects of 50% weight-bearing, ibandronate, and mechanical stimulation on bone structural properties measured using pQCT in 3-month-old Sprague-Dawley rats.

	Factor Effects (Significance)		
Property	50% Wt.-bearing	Ibandronate	Mech. stimulus
Cortical area	-6.9 (.01)	6.5 (.02)	6.5 (.06)
Polar S.M.A.	-12.7 (.002)	8.9 (.02)	12.1 (.03)
Polar section modulus	-1.8 (n.s.)	7.2 (.04)	7.9 (.08)
Trabecular density	-18.6 (.003)	21.8 (.001)	3.6 (n.s.)

Several observations are apparent from the ANOVA's shown in Table 6. First, 50% weight-bearing is not sufficient to prevent loss in bone strength and density – all effects are negative, and reach statistical significance in three out of four comparisons. Second, administration of ibandronate has a significant positive effect on all bone structural properties. The effect is particularly strong for trabecular density. Finally, mechanical stimulation through substrate vibration has a positive significant or near-significant ($p<.10$) effect on the three cortical bone geometric properties, although it has no significant effect on trabecular bone density.

These observations are reinforced through examination of individual treatment group trends through the experimental period (Fig. 5). When expressed relative to changes in controls (free-ranging rats) the negative effects of 50% weight-bearing on bone structural properties are clearly apparent (Fig. 5A). When ibandronate is administered to 50% weight-bearing rats, relative changes in properties are nonsignificantly different from those of controls (Fig. 5B). Mechanical stimulation produces the same effects for the cortical bone properties, but not trabecular bone density (Fig. 5C). Finally, ibandronate administered to free-ranging rats has a significant positive effect on trabecular bone density (Fig. 5D).

Thus, it appears that a partial weight-bearing environment (e.g., that on Mars, or artificially created during spaceflight) would not be sufficient to maintain bone strength. Mechanical stimulation through substrate vibration is effective in maintaining cortical bone strength, but apparently does not maintain trabecular bone density (which is proportional to trabecular bone strength (Carter and Hayes, 1977)). Therefore, a countermeasure involving mechanical stimulation through substrate vibration may need to be supplemented with a pharmacologic treatment to prevent loss of strength in all bone components.

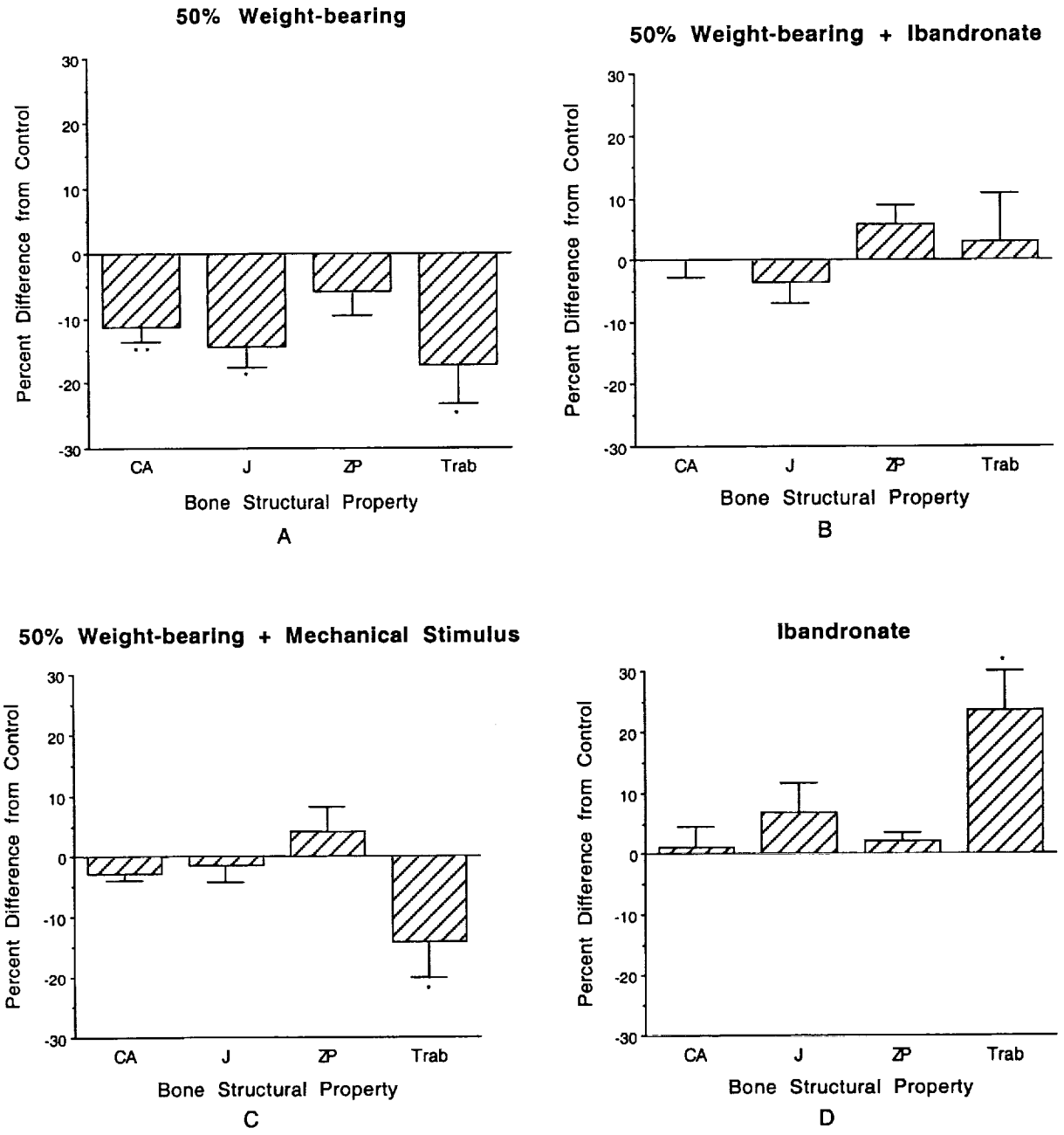


Figure 5. Percent change in rat experimental treatment groups relative to controls in four bone structural properties. * $p < .05$; ** $p < .01$, t tests with controls (free-ranging).

IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

Long-term exploration of space presents a number of formidable challenges, both technological and physiological. Loss of bone strength and increased risk of fracture, both during spaceflight and after return to Earth, are major concerns. To date, while the general problem has been recognized, specific risk assessments have not been carrried out. Also, the effectiveness of different countermeasures in reducing such risk have not been determined. Thus, two of the goals articulated in the Critical Research Path for Mars exploration, with respect to bone, have not previously been met.

The data collected in the present project begin to address these issues. Using hip scan data from Mir cosmonauts, both 2-D and 3-D mechanical models are used to estimate the actual risk of fracture after prolonged spaceflight. Hip fracture risk after 12 months exposure to microgravity is estimated to increase by almost 30% on average over baseline values, and including expected individual variability the range in this estimate is likely to be much greater. Thus, hip fracture (used here as a general index of fracture susceptibility) is in fact a significant concern during and after spaceflight. Studies using animal models indicate that a partial (.5 G) weight-bearing environment, on Mars or artificially created during spaceflight, will probably not be sufficient to maintain the strength of weight-bearing bones. Two potential countermeasures do appear to hold considerable promise, though. First, changing the vibrational characteristics of substrates in a partial weight-bearing environment can stimulate at least cortical bone formation and maintain cortical bone strength. However, this technique does not appear to be effective in maintaining trabecular bone strength. Administration of a bisphosphonate (in this study, ibandronate) does effectively maintain both cortical and trabecular bone. Given expressed concerns regarding invasive pharmacologic countermeasures, it may be that a combination of mechanical stimulation together with pharmacologic intervention when necessary represents the most feasible and acceptable solution.

Perhaps the most general conclusion that can be reached based on the results of this study is that bone structural assessment must be included in any attempt to monitor, interpret, and counteract bone loss during spaceflight. Changes in bulk measures of bone mass or density are insufficient to estimate fracture risk on an individual basis, and extrapolation from Earth-based models are not warranted. Direct measurement of bone structure while in space will be critical in long-term missions, to identify, treat, and follow up astronauts/cosmonauts whose fracture strength is found to decline significantly. Given the above estimates of fracture risk, this is likely to be a signficant issue for most of these individuals. Thus, the first recommendation here for future research is to develop a feasible method for measuring bone structural changes in space. A possible device (AMPDEXA) is currently under development within the NSBRI.

The second recommendation is to extend the results of the mechanical stimulation and ibandronate experiments to human trials, e.g., on the new Space Station, and to begin considering these factors (especially the substrate vibrational effects) in planning for design of future transport and/or habitation quarters during spaceflight. Additional studies using more permutations of the animal treatments reported here (i.e., different combinations of treatments) should also be carried out in the near future to more specifically define the optimal total treatment regimen. These studies should also be carried out in a larger animal model, e.g., dogs, whose bone physiology and mechanical properties more closely resemble those of humans.

Finally, in the near-term, structural data should continue to be collected from bone scans of cosmonauts and astronauts working and living on the new Space Station, before and after

spaceflight. These data are currently our best indicators of the effects of microgravity on human skeletal structure. Additional data will help to define the ranges of variation in human skeletal response to space. With additional data on individuals, it may also be possible to correlate loss in bone strength with other factors such as sex, age, and prior physical conditioning, to more precisely identify those individuals at the greatest risk.

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APPENDIX A: PROJECT RESEARCH DATA

Appendix Table 1: Bedrest Data

BMD and cross-sectional geometry values normalized to base-line on 6 bed-rest subjects

		Neck Values					Intertrochanteric Values				Shaft Values				
Subject #	Scan time point (days	BMD	Section Modulus	CSA	Width	Mean Cortical thickness	BMD	Section Modulus	CSA	Width	BMD	Section Modulus	CSA	Width	Mean Cortical thickness
1	Base-line	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
1	29	0.999	0.992	0.998	1.000	1.000	1.004	1.009	1.008	1.004	0.974	0.967	0.966	0.992	0.960
1	67	0.990	0.992	0.978	0.987	0.994	1.002	0.992	0.996	0.994	0.982	0.959	0.970	0.988	0.978
1	151	0.977	0.992	0.986	1.010	0.975	0.988	0.992	0.994	1.006	0.985	0.984	0.984	0.999	0.977
1	192	0.981	0.992	0.981	1.000	0.983	1.014	0.993	1.015	1.001	1.013	1.023	1.010	0.997	1.017
1	234	0.975	0.992	0.963	0.988	0.983	1.020	1.026	1.022	1.002	1.007	1.004	1.000	0.992	1.011
2	base-line	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
2	31	0.998	0.992	1.008	1.010	0.999	0.976	0.965	0.972	0.996	0.983	0.965	0.974	0.990	0.981
2	66	0.982	0.992	0.971	0.988	0.981	0.971	1.010	0.986	1.015	0.965	0.981	0.973	1.008	0.953
2	117	0.953	0.992	0.961	1.009	0.957	0.952	0.957	0.959	1.008	0.959	0.962	0.960	1.000	0.945
2	150	0.940	0.992	0.941	1.001	0.934	0.948	0.935	0.952	1.005	0.946	0.973	0.959	1.014	0.924
2	192	0.954	0.992	0.951	0.997	0.952	0.958	0.936	0.959	1.000	0.980	0.992	0.987	1.007	0.968
2	239	0.972	0.992	0.967	0.995	0.957	0.957	0.897	0.947	0.990	0.997	0.978	0.988	0.991	0.999
2	311	0.927	0.992	0.947	1.021	0.916	0.931	0.900	0.933	1.002	0.943	0.944	0.948	1.005	0.919
2	352	0.936	0.992	0.940	1.004	0.928	0.918	0.866	0.917	0.999	0.950	0.980	0.970	1.021	0.924
3	base-line	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
3	32	1.004	1.023	1.022	1.018	1.000	0.998	1.005	1.006	1.008	1.001	1.032	1.002	1.001	0.999
3	107	0.924	0.950	0.936	1.013	0.915	0.935	0.936	0.947	1.013	0.975	0.988	0.972	0.996	0.975
3	149	0.933	0.976	0.955	1.023	0.924	0.935	0.965	0.953	1.018	0.974	0.986	0.969	0.995	0.966
3	165	0.928	1.005	0.957	1.031	0.929	0.947	0.922	0.958	1.012	0.984	0.992	0.983	0.999	0.978
3	205	0.920	0.932	0.926	1.007	0.911	0.950	0.964	0.963	1.014	0.989	1.002	0.984	0.994	0.989
3	247	0.928	0.996	0.949	1.022	0.917	0.960	0.967	0.974	1.015	0.988	1.021	0.995	1.007	0.982
3	319	0.979	1.008	0.993	1.014	0.970	0.982	0.987	0.990	1.008	0.991	1.024	0.997	1.006	0.986
4	base-line	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
4	29	0.993	0.991	1.002	1.009	0.989	1.012	1.040	1.015	1.002	0.976	0.978	0.976	1.000	0.977

4	65	1.009	1.028	1.025	1.015	1.011	1.020	0.993	1.015	0.995	0.977	0.967	0.987	1.010	0.972
4	109	0.952	0.949	0.956	1.004	0.943	0.977	1.014	0.985	1.008	0.942	0.949	0.947	1.005	0.929
4	147	0.972	1.014	0.996	1.025	0.954	0.981	0.951	0.969	0.988	0.940	0.915	0.937	0.997	0.927
4	211	0.926	0.929	0.958	1.035	0.912	0.963	0.984	0.964	1.002	0.957	0.979	0.968	1.012	0.942
5	Base-line	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
5	32	0.973	0.958	0.963	0.989	0.974	0.977	0.999	0.979	1.002	0.962	0.965	0.961	1.000	0.948
5	67	0.999	1.030	1.014	1.015	1.006	0.956	0.937	0.959	1.003	0.984	0.990	0.989	1.005	0.979
5	106	0.970	0.978	0.973	1.003	0.974	0.920	0.914	0.927	1.007	0.960	0.971	0.972	1.012	0.948
5	148	0.973	0.996	0.978	1.005	0.960	0.939	0.911	0.943	1.004	0.982	1.014	1.006	1.025	0.969
5	167	0.950	0.966	0.951	1.001	0.945	0.939	0.940	0.939	1.000	0.963	0.988	0.983	1.021	0.955
5	213	0.978	0.990	0.979	1.001	0.981	0.956	0.923	0.954	0.997	0.987	1.016	1.008	1.021	0.976
5	247	0.990	1.023	1.000	1.009	0.981	0.946	0.910	0.947	1.000	0.970	0.990	0.989	1.020	0.959
5	290	0.958	1.000	0.977	1.020	0.952	0.951	0.936	0.954	1.004	0.969	0.996	0.984	1.015	0.959
5	354	0.973	0.997	0.985	1.012	0.974	0.954	0.976	0.960	1.006	0.958	0.991	0.975	1.018	0.948
5	431	0.978	1.010	0.990	1.011	0.988	0.956	0.959	0.963	1.007	0.966	0.992	0.981	1.016	0.955
5	546	0.986	1.046	1.009	1.023	0.985	0.975	0.982	0.971	0.996	0.952	0.972	0.959	1.008	0.938
6	Base-line	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
6	29	1.005	0.992	0.997	0.992	0.992	0.988	0.971	0.995	1.007	0.976	0.962	0.970	0.993	0.960
6	65	0.993	0.986	0.987	0.993	0.983	0.986	0.965	0.988	1.002	0.975	0.944	0.963	0.988	0.957
6	109	0.986	0.970	0.986	1.000	0.975	0.967	0.906	0.959	0.991	0.949	0.925	0.944	0.994	0.920
6	159	0.962	0.974	0.985	1.024	0.944	0.957	0.900	0.950	0.993	0.970	0.943	0.965	0.996	0.947
6	227	0.999	0.958	0.990	0.991	0.989	0.988	0.940	0.973	0.985	0.975	0.927	0.952	0.977	0.964
6	268	0.989	1.040	1.013	1.025	0.975	0.982	0.937	0.981	0.999	0.980	0.961	0.980	1.000	0.960

Appendix Table 2: Mir Cosmonaut Data

Normalized post flight data — Percent change per month

Cosmonaut #	Flight months	N BMD	N CSA	N width	N mean cortex	N Z	T BMD	T CSA	T width	T Lat Z	S BMD	S CSA	S width	S mean cortex	Shaft Z
1	6.0	-0.23%	-0.23%	0.00%	-0.19%	-0.65%	-1.31%	-1.29%	0.03%	-1.62%	-0.51%	-0.51%	0.00%	-0.66%	-0.54%
2	6.0	-1.42%	-0.89%	0.58%	-1.51%	-1.42%	-1.19%	-1.78%	-0.63%	-2.61%	-0.31%	-0.48%	-0.18%	-0.24%	-0.87%
2	6.3	-1.97%	-1.89%	0.09%	-2.11%	-1.89%	-0.49%	-0.60%	-0.11%	-0.29%	-0.11%	-0.16%	-0.05%	-0.25%	-0.30%
3	4.4	-0.17%	-0.23%	-0.06%	0.00%	-0.32%	-1.84%	-1.67%	0.19%	-1.51%	-1.08%	-1.37%	-0.31%	-1.32%	-1.23%
3	6.0	-0.20%	-0.76%	-0.57%	-0.10%	-1.42%	0.23%	0.55%	0.31%	1.51%	-0.31%	-0.25%	0.06%	-0.45%	0.03%
4	4.4	-1.54%	-0.33%	1.31%	-1.70%	0.87%	-1.59%	-1.67%	-0.08%	-3.43%	-1.43%	-1.23%	0.21%	-1.67%	-1.41%
5	5.8	-1.87%	-1.65%	0.24%	-2.01%	-1.73%	-2.11%	-1.85%	0.30%	-1.42%	-1.19%	-0.93%	0.28%	-1.54%	-0.99%
5	6.1	-1.60%	-1.78%	-0.19%	-1.73%	-2.15%	-1.21%	-1.06%	0.17%	-0.30%	-1.02%	-0.98%	0.04%	-1.27%	-0.92%
6	4.8	-1.81%	-1.64%	0.18%	-2.14%	-1.02%	-1.10%	-0.78%	0.34%	0.66%	0.05%	-0.22%	-0.26%	0.29%	-0.54%
7	5.8	-1.19%	-1.01%	0.20%	-1.25%	-0.98%	-1.05%	-0.66%	0.42%	-0.28%	-1.21%	-0.84%	0.40%	-1.51%	-0.39%
8	10.4	-1.80%	-1.26%	0.65%	-2.01%	-1.38%	-1.48%	-1.65%	-0.20%	-1.54%	-0.97%	-0.64%	0.37%	-1.12%	-0.81%
9	5.8	1.73%	-0.43%	-1.96%	2.46%	0.25%	-0.57%	-0.51%	0.06%	-0.66%	-0.82%	-0.71%	0.12%	-1.09%	-0.72%
10	4.8	-1.34%	-1.03%	0.33%	-1.30%	-0.57%	-2.20%	-2.20%	0.00%	-2.97%	-0.41%	-0.64%	-0.23%	-0.44%	-1.12%
10	6.6	-1.45%	-1.48%	-0.04%	-1.41%	-1.66%	-1.73%	-1.90%	-0.19%	-2.13%	-1.34%	-1.30%	0.04%	-1.66%	-0.96%
14	4.2	-2.46%	-2.00%	0.52%	-2.76%	-1.97%	-1.96%	-2.18%	-0.24%	-1.95%	-0.25%	-0.11%	0.14%	-0.35%	-0.29%
17	4.9	-2.19%	-0.29%	2.13%	-2.07%	-0.92%	-1.76%	-1.92%	-0.17%	-1.10%	-0.98%	-0.56%	0.44%	-1.31%	-0.34%
17	5.6	-1.48%	-1.34%	0.15%	-1.61%	-1.38%	-1.68%	-2.02%	-0.37%	-2.07%	-1.06%	-0.71%	0.38%	-1.34%	-0.76%
18	6.3	-1.41%	-1.29%	0.13%	-1.35%	-0.99%	-1.02%	-1.00%	0.03%	-1.11%	-0.14%	-0.42%	-0.29%	0.10%	-0.58%
18	6.0	-3.79%	-3.61%	0.24%	-1.85%	-4.08%	-3.57%	-3.59%	-0.03%	-4.60%	-0.51%	-1.64%	-1.16%	0.23%	-1.68%
20	6.6	-2.38%	-3.47%	-1.29%	-2.49%	-3.43%	-3.02%	-2.48%	0.67%	-1.84%	-1.57%	-1.19%	0.42%	-1.94%	-1.00%
21	6.1	-2.72%	-2.55%	0.20%	-2.82%	-2.09%	-1.24%	-1.66%	-0.46%	-3.20%	-0.79%	-1.19%	-0.42%	-0.93%	-1.30%
21	6.5	-1.23%	-0.96%	0.34%	-1.38%	-1.27%	-1.76%	-1.57%	0.26%	-1.13%	-0.56%	-0.52%	0.05%	-0.80%	-0.24%
22	6.6	0.82%	0.20%	-0.59%	1.35%	0.12%	0.15%	0.23%	0.07%	0.50%	-0.63%	-0.75%	-0.13%	-0.68%	-0.84%
27	6.0	-1.23%	-0.84%	0.42%	-1.39%	-1.01%	-1.26%	-1.26%	0.00%	-0.84%	-0.60%	-0.60%	0.00%	-1.11%	-0.56%
28	5.6	-0.64%	-0.59%	0.06%	-0.90%	-1.43%	-2.13%	-2.05%	0.10%	-2.32%	-1.16%	-1.06%	0.12%	-1.36%	-0.59%
36	6.6	-0.58%	-0.80%	-0.23%	-0.65%	-0.82%	-0.77%	-0.77%	0.00%	-0.45%	-0.69%	-0.61%	0.09%	-0.86%	-0.73%

N: mid-femoral neck; T: intertrochanteric; S: proximal femoral shaft
CSA: cross-sectional area; mean cortex: mean cortical thickness; Z: section modulus

Appendix Table 3: Tail-Suspended Rat Data

Treatment	ID	CA1	CA2	J1	J2	Zp1	Zp2	Tden1	Tden2
FR	Rat3 humerus	4.11	4.42	3.59	4.21	2.39	2.48	431.1	405.7
FR	Rat10 humerus	3.53	3.96	2.75	3.45	1.97	2.09	531.1	457.7
FR	rat16 humerus	3.68	3.92	2.59	3.02	1.9	2.1	458.6	435.6
FR	Rat1.27 humerus	3.67	3.93	2.81	3.15	2.06	2.18	430.6	397.5
FR	Rat2.1 humerus	3.52	3.94	2.43	3.04	1.9	2.04	541.4	474.4
FR	Rat2.17 humerus	3.64	3.77	2.76	2.76	1.86	1.93	437.7	445.2
FR	Rat2.18 humerus	3.83	3.62	2.91	2.66	2.23	2.05	489	513.1
FR	Rat2.19 humerus	3.59	4.18	2.83	3.46	2.17	2.54	377.1	380.8
50%	Rat8 humerus	3.96	3.62	3.67	3.29	2.7	2.3	408.9	348
50%	Rat13 humerus	3.88	3.63	3.5	3.3	2.57	2.33	401.8	274.3
50%	Rat1.28 humerus	4.24	4.04	4.17	3.87	2.81	2.58	490.3	409.2
50%	Rat2.2 humerus	3.94	4.1	2.96	3.19	1.99	2.09	611.8	446.2
50%	Rat3.16.00 humerus	4.65	4.73	5.37	5.22	2.92	3.23	651	603.4
50%	Rat 3.17.00 Humerus	4.22	4.1	3.69	3.59	2.53	2.56	423.3	496.8
50%	Rat4.6.00b Humerus	5.08	4.06	6.21		2.57		378.9	212.3
50%	Rat4.7.00 Humerus	5.03	4.45	4.88		3.03		227	227
50%	Rat4.8.00 Humerus	4.95	4.98	4.24		2.57		425.4	266
50%	Rat4.8.00-2 Humerus	4.68	5.05	5.17	6	3.18	3.69	448.7	266.6
50%	Rat4.9.00 Humerus	4.09	4.01	3.42	3.4	2.46	2.44	429.4	260.5
50%+Iban.	Rat11 humerus	3.8	3.92	3.95	4.09	2.74	2.72	364.6	422.7
50%+Iban.	Rat9 humerus	4.03	4.85	3.88	3.99	2.28	2.74	575.8	509.2
50%+Iban.	rat23 humerus	4.33	4.27	3.81	3.8	2.44	2.49	502.1	506.4
50%+Iban.	rat24 humerus	4.17	4.7	4.01	4.93	2.65	3.06	388.9	429.7
50%+Iban.	Rat3.18.00 Humerus	4.37	4.7	3.92	4.54	2.5	3	338.6	369.3
50%+Iban.	Rat4.17.00 Humerus	4.05	4.21	3.26	3.6	2.3	2.5	383.7	
50%+Iban.	Rat7.28.00 humerus	4.29	4.54	3.65	4.21	2.55	2.92	442.7	289.7
50%+3HZ	Whit3.3.11 humerus	4.15	4.42	3.31	3.76	2.31	2.53	418.3	388.5
50%+3HZ	Whit3.3.12b humerus	4.27	4.4	3.67	3.94	2.64	2.83	524.5	479.3
50%+3Hz	Rat3.4.13 humerus	3.7	4.05	2.95	3.6	2	2.59	384.7	250.4
50%+3Hz	Rat3.4.14 humerus	3.71	3.84	3.36	3.56	2.34	2.5	335.9	200
50%+3Hz	Rat7.17.00b Humerus	4.37	4.59	4.82	5.42	3.05	3.14	457.1	465
50%+3Hz	Rat7.21.00 Humerus	4.05	4.18		3.69		2.37	394	276.3
50%+3Hz	Rat7.27.00 Humerus	4.39	4.44	4.6	4.88	2.94	3.03	370.7	323.3
FR+Iban.	Rat2.11 humerus	3.76	4.72	3.35	4.53	2.37	2.6	482.5	446.6
FR+Iban.	Rat2.12 humerus	4.11	4.78	3.86	4.87	2.7	3.02	379.4	508.9
FR+Iban.	Rat2.15 humerus	3.73	3.97	3.42	3.8	2.51	2.67	363.1	493.9
FR+Iban.	Rat2.16 humerus	4.64	4.73	5	5.39	3.16	3.29	398.6	476.6
FR+Iban.	Rat7.13.00b Humerus	4.15	4.32		3.8		2.68		
FR+Iban.	Rat8.3.00 humerus	4.31	4.45	3.81		2.5		301	341.9
FR+Iban.	Rat8.4.00 Humerus	4.32	4.43	3.88	4.73	2.2	2.36	429.4	505

CA: cortical area; J: polar second moment of area; Zp: polar section modulus; Tden: trabecular density
1: measurement at beginning of treatment period; 2: measurement at end of treatment period

APPENDIX B: LIST OF PUBLICATIONS SUPPORTED THROUGH NSBRI

Allen, MR, Schultheis, L, Ruff, CB, Hogan, HA, Shapiro, JR, and Bloomfield, SA. 1999. Ibandronate increases femoral neck bone mineral content, area, and ultimate strength in growing rats. Am. Coll. Sports Med. Ann. Meeting.

Dapthary, MM, Shapiro, JR, Ruff, CR, Ruiz, J, Bloomfield, SA, and Schultheis, L. 2000. Effects of partial weightbearing, dynamic loading and ibandronate as countermeasures to bone loss in the suspended rat. The 13th Humans in Space Symposium "Exploring Space", Santorini, Greece.

Newman, D, Schaffner, G, Oden, ZM, Ruff, C, and Beck, T. 2000. Dynamic modeling to assess hip loading for Martian space exploration. Biomed. Eng. Soc. Ann. Meeeting, Seattle.

Oden, Z, Schaffner, G, Beck, T, Ruff, C, Biegler, F, Wang, W, and Newman, D. 2000. Strength reductions in the femur following long-term space flight: A finite element simulation. Am. Soc. Bone Min. Res., Toronto.

Ruff, CB, Beck, TJ, LeBlanc, AD, Evans, HJ, Shackelford, L, Oganov, V, and Schneider, VS. 1999. Effects of prolonged weightlessness on hip structural geometry in Russian cosmonauts. Am. Soc. Bone. Min. Res., St. Louis.

Schaffner, G. 1999. Assessment of Hip Fracture Risk in Astronauts Exposed to Long-Term Weightlessness. Ph.D. Thesis, MIT.

Schaffner, G, and Newman, D. 2000. Computation of astronaut hip joint forces during locomotion and falls in Earth and Mars gravity. Aerospace Med. Assn. 71st Ann. Sci. Meeting, Houston.

Schultheis, L, Rastogi, S, Ruff, C, Bloomfield, S, and Shapiro, J. 1999. Partial weightbearing with a force spectrum typical of normal ambulation improves bone strength in hindquarter suspended rats. Orthop. Res. Soc., Orlando.

Schultheis, L, Ruff, CB, Bloomfield, SA, Hogan, HA, Fedarko, N, Thierry-Palmer, M, Ruiz, J, and Shapiro, JR. 1999. Disuse bone loss in hindquarter suspended rats: Partial weightbearing, exercise and ibandronate treatment as countermeasures. Am. Soc. Bone Min. Res., St. Louis.

NSBRI RESEARCH PROGRAM
CARDIOVASCULAR ALTERATIONS

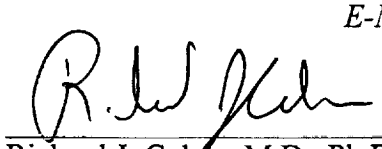
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Cohen, R. J.	CO-I	MIT	
Ramsdell, C. D.	CO-I	Harvard	
Sheynberg, N.	CO-I	Harvard	
Cohen, R. J.	**PI**	**MIT**	**Alterations in Cardiovascular Regulation and Function During Simulated Microgravity**
Ramsdell, C. D.	CO-I	Harvard	
Sherman, D. A.	CO-I	MIT	
Williams, G. H.	**PI**	**Harvard**	**Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity**
Ramsdell, C. D.	CO-I	Harvard	
Sheynberg, N.	CO-I	Harvard	
Shoukas, A. A.	**PI**	**Hopkins/SOM**	**Rodent Studies of Cardiovascular Deconditioning**
Berkowitz, D.	CO-I	Hopkins/SOM	
Kamm, R. D.	**PI**	**MIT**	**Computational Models of the Cardiovascular System and Its Responses to Microgravity**
Mark, R. G.	CO-I	MIT	
Schneider, M. D.	**PI**	**Baylor**	**Cardiac Atrophy**
Abdellatif, M.	CO-I	Harvard	
Lorell, B.H.	CO-I	Harvard	
Cohen, R. J.	**PI**	**MIT**	**Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias During Simulated Microgravity**
Ramsdell, C. D.	CO-I	Harvard	
Sherman, D. A.	CO-I	MIT	

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Program Report

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PROGRAM EXECUTIVE SUMMARY

The cardiovascular system seems to function remarkably well during conditions of space flight. However, particularly during prolonged space flight, the process of cardiovascular deconditioning impairs the cardiovascular system's ability to readapt to a gravity environment. Upon reentry from space flight into the Earth's gravitational field, astronauts experience orthostatic hypotension and reduced exercise capacity, which limits their ability to function during reentry and after landing. For example, in many cases, the orthostatic hypotension is sufficiently severe that astronauts cannot stand erect for some time after landing, thus precluding emergency egress on Earth or another planetary surface. Despite years of research, the mechanisms leading to orthostatic intolerance following microgravity exposure remain poorly characterized and current countermeasures are not adequately effective.

One aspect of cardiovascular deconditioning is a reduction in cardiac mass, the mechanism of which is not known, nor are its functional correlates and reversibility known. In addition, there is significant anecdotal evidence that space flight is associated with decreased cardiac electrical stability which may pose a life threatening risk to astronauts. For example, one crew member during the Skylab missions had a five beat run of ventricular tachycardia during lower body negative pressure. Furthermore, analysis of nine 24 hour Holter monitor recordings obtained during long term space flight on Mir revealed one 14 beat run of ventricular tachycardia. Possible mechanisms of arrhythmias and countermeasure strategies have barely been addressed. As long duration missions and older astronauts become more common, alterations in cardiovascular function resulting space flight are more likely to have an impact on mission success and astronaut safety. Thus, it becomes imperative to understand mechanisms of cardiovascular deconditioning and to develop appropriate countermeasures. The death of an experimental primate shortly after return from space, with cardiovascular mechanisms suspected as primary or contributing causes, lends urgency to these objectives.

The objective of this research program is to apply the most powerful technologies available to determine, in ground-based studies, the mechanisms by which space flight affects cardiovascular function, and then on the basis of an understanding of these mechanisms to develop rational and specific countermeasures.

The research effort is divided among seven projects:

A. Human Studies Core — Gordon H. Williams, PI
This project revolves around a 16 day head down tilt bed-rest study that examines the mechanisms by which bed-rest and disruption of circadian rhythms affect cardiovascular hemodynamic regulation and cardiac electrical stability.

B. Alterations in Cardiovascular Regulation and Function During Simulated Microgravity — Richard J. Cohen, PI
This study involves the application of a number of powerful new non-invasive measurement technologies, including cardiovascular system identification (CSI) for the assessment of closed-loop cardiovascular regulation in order to understand mechanisms involved in the development of orthostatic intolerance during microgravity simulated by bed-rest and to develop effective countermeasures.

C. Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity — Gordon H. Williams, PI
This project studies alterations in the responsivenes of the renin-angiotensin-aldosterone hormonal salt and fluid regulatory system in response to the head down tilt bed-rest model of weightlessness. The goal of the project is to understand how these systems lead to the development of orthostatic hypotension and to develop effective countermeasures.

D. Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias During Simulated Microgravity — Richard J. Cohen, PI
This study involves measurement of microvolt level T wave alternans measure of before and after bed-rest in order to determine whether simulated microgravity alters cardiac electrical stability..

E. Cardiovascular Deconditioning in Rodents — Artin A. Shoukas, PI
Mechanisms involved in the development of orthostatic intolerance are studied in the tail suspended rodent model while taking advantage of the more invasive measurements that can be made in the rodent model as compared to the human model. The rodent model may also serve as a platform to test potential countermeasures before they are evaluated in human studies.

F. Computational Models of the Cardiovascular System — Roger Kamm, PI
In this project, a computer model that simulates the critical components and behaviors of the cardiovascular system is being developed. The goal of this project is to understand how microgravity alters cardiovascular function and leads to the development of orthostatic hypotension. This model is validated using the data collected in the rodent and human studies and used to test potential countermeasures.

G. Cardiac Atrophy — Michael Schneider, PI
The objective of this project is to determine the cellular and genetic mechanisms by which cardiac mass is reduced during space flight and to develop appropriate countermeasures using a unique rodent model of cardiac unloading.

These studies address the major cardiovascular problems associated with space flight. The plan, in each case, is first to determine the basic mechanisms of the cardiovascular alterations and then, on the basis of the understanding of these mechanisms, to propose and test rational, specific countermeasures. For the current year these studies are mandated to involve only ground-based studies, but as described below, we plan to develop proposals for flight experiments as well.

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PROGRAM RESEARCH ACCOMPLISHMENTS

Space flight is associated with a constellation of cardiovascular deconditioning effects including decreased orthostatic tolerance and exercise capacity upon return to normal gravity and decreased cardiac muscle mass and an occurrence of a variety of arrhythmias in some individuals during space flight. Maintenance of exercise capacity and orthostatic tolerance at pre-flight levels requires the integrity of both cardiac pump function and the multiple neurohumoral control mechanisms that mediate the hemodynamic response to these challenges. The documented decreases in orthostatic tolerance and exercise capacity following space flight imply microgravity induced alterations in integrated hemodynamic regulation. Recent evidence demonstrates that several long held consensus hypotheses about some of the most basic physiologic changes in space, *e.g.*, the direction of change in central venous pressure following the cephalad fluid shift and the renal-endocrine response to the cephalad fluid shift, were in fact incorrect. Such findings demonstrate that, while many changes in cardiovascular function resulting from space flight have been documented, what is lacking is a complete understanding of integrated cardiovascular function during and following microgravity exposure. The general objective of these investigations are to elucidate the mechanisms responsible for these microgravity induced alterations by applying the most powerful technologies available and to develop rational, specific countermeasures against the deleterious effects of space flight on the cardiovascular system. Specific objectives include:

- To determine the mechanisms by which microgravity and disruption of circadian rhythms lead to the development of orthostatic intolerance following space flight.
- To quantify changes in cardiac conduction processes that may lead to an enhanced susceptibility to arrhythmias during space flight.
- To develop a computer model capable of integrating the multiple effects of space flight on cardiovascular regulation and predicting the likely effect of countermeasures.
- To study the molecular and cellular basis of space flight induced cardiac atrophy.

Powerful new quantitative techniques are used in these studies to define physiologic alterations and the effects of countermeasures. These include Cardiovascular System Identification (CSI), the measurement of microvolt level T-Wave Alternans (TWA), and the most modern techniques in animal physiology, molecular biology, and computer simulation. CSI, is a non-invasive technique for assessing closed-loop cardiovascular regulation by means of mathematical analysis of second-to-second fluctuations in physiologic signals to assess closed-loop cardiovascular hemodynamic regulation. TWA is a non-invasive means of assessing susceptibility to ventricular arrhythmias and sudden death bymeasurement of microvolt level fluctuations T wave morphology. Because CSI and TWA are non-invasive and require only minutes for data collection, they are ideally suited to the eventual study of astronaut subjects during space flight as well as having important applications for clinical medicine on earth.

The Cardiovascular Alterations Team investigations are all closely coordinated, with extensive sharing of data between investigators and with particular attention to using the experimental data

in the computer modeling effort. We also have a flexible research strategy where research efforts can be redirected as new information is developed.

Brief Description of Projects Within Program and Their Accomplishments

The Cardiovascular Alterations Team was organized into seven projects:

A. Human Studies Core — Gordon H. Williams, PI
- Human head down tilt bed-rest is a well established ground-based model of microgravity . The objective of this project, which was successfully implemented, was to establish and run a rigorously controlled 16 day head-down tilt bed-rest protocol in the clinical research center at the Brigham and Women's hospital with tight regulation of diet and environmental factors. This protocol supported specific studies B. and C listed below.

B. Alterations in Cardiovascular Regulation and Function During Simulated Microgravity — Richard J. Cohen, PI
- This study involves analysis of mechanisms involved in the development of orthostatic intolerance by applying a powerful new non-invasive technology, developed with NASA support, Cardiovascular System Identification (CSI), to subjects in a head-down tilt bed-rest study. CSI, is a non-invasive technique for assessing closed-loop cardiovascular regulation by means of mathematical analysis of second-to-second fluctuations in physiologic signals to assess closed-loop cardiovascular hemodynamic regulation.

- This study demonstrated a number of alterations in closed-loop regulatory mechanisms associated with exposure to simulated microgravity.

- Based on the results of the initial studies, and incorporating results from animal studies and computer simulations in projects E and F below, the alpha agonist midodrine administered at the very end of bed-rest was proposed and tested as a countermeasure to the development of orthostatic intolerance. The results indicated that midodrine is in fact a very effective countermeasure.

C. Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity — Gordon H. Williams, PI
- This project studied alterations in the responsivenes of the renin-angiotensin-aldosterone hormonal salt and fluid regulatory system in respons to the head down tilt model of weightlessness.

- Augmented responsiveness of the renin-angiotensin and aldosterone systems was demonstrated as a result of exposure to microgravity.

- The development of orthostatic intolerance tended to occur in those individuals who least effectively augmented the responsiveness of the renin-angiotensin-aldosterone systems.

D. Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias During Simulated Microgravity — Richard J. Cohen, PI

- This study utilized a new non-invasive technology developed with NASA support, the measurement of microvolt level T-wave alternans to assess alterations in cardiac electrical stability resulting from 16 days of bed-rest.

- This study found that even 16 days of bed rest led to an increased incidence of microvolt level T wave alternans indicating that simulated microgravity alters cardiac electrical processes. Presence of T-wave alternans in clinical patient populations has been shown to be indicative of increase risk of ventricular arrhythmias.

- The T-Wave Alternans technology has been successfully commercialized and cleared by the FDA as a means of identifying patients at risk of ventricular arrhythmias and sudden cardiac death. T-Wave Alternans has been demonstrated in multiple international trials to be exceedingly effective and is now in clinical use to reduce the incidence of sudden cardiac death which is responsible for 1 in 7 of all deaths in the United States.

E. Cardiovascular Deconditioning in Rodents — Artin A. Shoukas, PI

- The rat-tail suspension model of simulated microgravity was used to study the effects of microgravity on hemodynamic regulation and to test countermeasures. The rat model provides an efficient, low-cost model in which extensive physiologic data can be obtained and various countermeasures evaluated.

- Tail suspension has been demonstrated to lead to impaired cardiac output responses to orthostatic challenges. This impaired response is associated with a diminished intravascular volume, and decreased responsiveness of the arterial and venous systems.

- A prototype "milking" external G-suit device which propels blood from the lower extremity to the thorax has been developed to reduce the development of orthostatic hypotension.

F. Computational Models of the Cardiovascular System — Roger Kamm, PI

- The goal of this project was to develop a computer model of cardiovascular regulation to study the effects of microgravity on the development of orthostatic intolerance. This model has been successfully developed and tested.

- A database of hemodynamic data from ground based and space flight studies have been assembled from a variety of investigators to develop and test the model.

- The model has been used to test hypotheses regarding the cardiovascular changes associated with microgravity and it has been demonstrated that a combination of adaptations are required in order to account for the experimental data.

- It was determined by computer simulations that the alpha-agonist midodrine would be a good candidate to be an effective countermeasure for the development of orthostatic intolerance.

G. Cardiac Atrophy — Michael Schneider, PI

- The objective of this project is to determine the cellular and genetic mechanisms by which cardiac mass is reduced during space flight and to develop appropriate countermeasures.

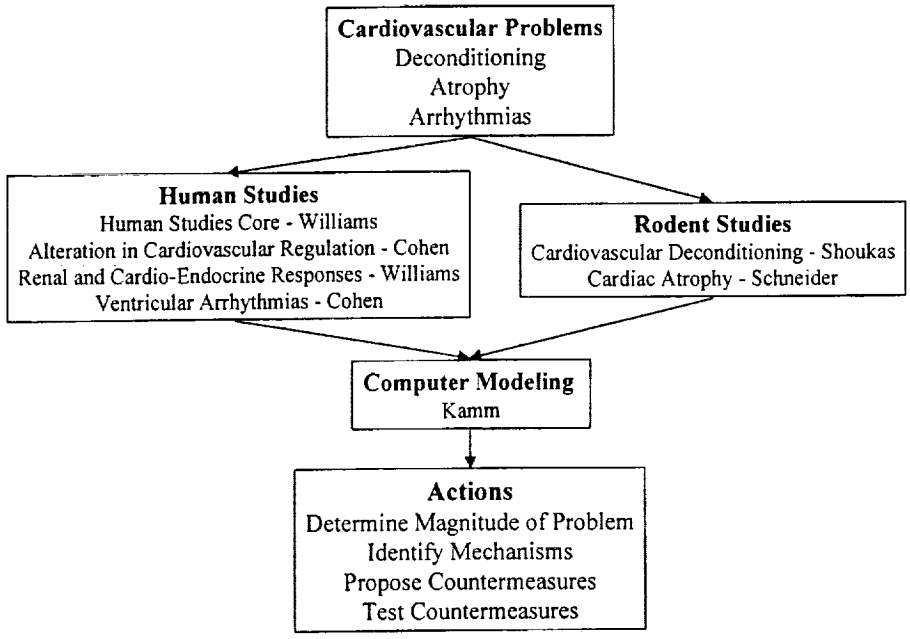
- A unique rodent model of cardiac unloading involving heterotopic transplantation of the heart into the abdominal cavity of a syngeneic mouse was established as well as microphysiologic techniques of assessing cardiac structure and function in this model. It was established that unloaded hearts demonstrated cardiac atrophy and diminished contractile reserve, and associated changes in gene expression were documented.

- It was demonstrated that growth hormone appears to be effective in diminishing the loss in cardiac mass and loss in contractile reserve.

General Program Strategy

The overall research strategy was to apply the optimal technology and expertise to study the effects of simulated space flight on alterations in cardiovascular structure and function in order to: 1) characterize quantitatively the nature and extent of each alteration; 2) prioritize the importance of each alteration for potential impact on astronauts' health and ability to function; 3) determine the physiologic mechanisms by which space flight induces the alteration in question; and, 4) propose and develop specific rational countermeasures and quantitatively evaluate their potential effectiveness.

The figure below illustrates the relationship between the various projects.



The investigators in the various projects interacted effectively on an individual basis, during team teleconferences, and semi-annual face-to-face team meetings and retreats.

RISK REDUCTION ACHIEVED BY PROGRAM

The program was quite successful in making progress in risk reduction.

Space Flight Risk Reduction

Orthostatic Intolerance Risk

- The human, animal and modeling studies improved our understanding of mechanisms leading to the development of post flight orthostatic intolerance. This improved understanding led to the identification of the alpha-agonist midodrine administered at the very end of the period of microgravity as a potential countermeasure. This countermeasure was then successfully tested in a bed-rest protocol and found to be very effective in preventing the development of orthostatic intolerance. Future work will focus on studying the effectiveness of midodrine in women as well as men, and in conducting space flight experiments for this countermeasure.

- The technology of Cardiovascular System Identification was established as an effective means of studying and monitoring closed-loop hemodynamic function before, during and after exposure to microgravity.

Ventricular Arrhythmia Risk

- It was demonstrated that even 16 days of bed rest led to the development of microvolt level T wave alternans indicating an alteration in cardiac electrical activity suggestive of increased susceptibility to ventricular arrhythmias. Future studies will focus on studying the effects of gender and age on the development of microvolt level T wave alternans in ground based studies and in flight experiments. If future studies indicate that space flight does increase susceptiblity to ventricular arrhythmias, a potential countermeasure, aldosterone blocking agents, has been identified for testing.

- The technology of T-wave alternans testing was established as a means of monitoring astronauts for changes in cardiac electrical stability.

Cardiac Atrophy Risk

- Cardiac unloading has been confirmed to decrease cardiac mass and contractilve reserve. Growth hormone has been identified as a potential countermeasure.

Risk Reduction on Earth (Spinoff Technologies which Benefit Health on Earth)

- T-wave alternans which was developed under NASA support has been successfully commercialized and FDA approved for identification of individuals at risk for ventricular arrhythmias and sudden cardiac death. It has been successfully tested in a multiple international clinical trials and is currently in increasing clinical use. This technology promises to have a major impact in reducing sudden cardiac death, which accounts for one in seven of all deaths in the United States.

- Cardiovascular System Identification has been demonstrated to be an effective technology for assessing closed-loop cardiovascular regulation and promises to be a powerful new tool for studying and monitoring patients with diseases that affect cardiovascular and neurologic function. These diseases include heart failure, diabetes and hypertension.

- The insights developed for understanding mechanisms of, and developing countermeasures for, orthostatic intolerance, ventricular arrhythmias, and cardiac atrophy associated with space flight will contribute to understanding and treating disease processes which lead to these same phenomena on earth.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

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HUMAN STUDIES CORE

Executive Summary

Major cardiovascular problems, secondary to cardiovascular deconditioning, may occur on extended space missions. While it is generally assumed that the microgravity state is the primary cause of cardiovascular deconditioning, sleep deprivation and disruption of diurnal rhythms may also play an important role. Factors that could be modified by either or both of these perturbations include: autonomic function and short-term cardiovascular reflexes, vasoreactivity, circadian rhythm of cardiovascular hormones (specifically the renin-angiotensin system) and renal sodium handling and hormonal influences on that process, venous compliance, cardiac mass, and cardiac conduction processes. The purpose of the Human Studies Core is to provide the infrastructure to conduct human experiments that allow the assessment of the likely role of such factors in the space travel associated cardiovascular deconditioning process, and to develop appropriate countermeasures. The Core takes advantage of the General Clinical Research Center at the Brigham and Women's Hospital, Boston, Massachusetts, to perform these studies.

The Core includes two general experimental protocols. The first protocol involves a head down tilt bed-rest study to simulate microgravity. The second protocol includes the addition of sleep deprivation to the simulated microgravity environment. Before and after each of these environmental manipulations, the subjects undergo acute stressors simulating changes in volume and/or stress, which could occur in space and on return to Earth. The subjects are maintained in a rigidly controlled environment with fixed sleep cycles, activity pattern, and dietary intake of nutrients, fluids, ions and calories.

Within the Core experimental protocol framework, investigators perform specific experiments, some based on the application of new non-invasive measurement techniques, to determine the effect of the environmental modifications on the status and responsiveness of the cardiovascular, endocrine, and renal homeostatic systems. In the project led by Professor Cohen, titled Alterations in Cardiovascular Regulation and Function during Simulated Microgravity, investigators apply cardiovascular system identification (CSI) techniques to characterize important cardiovascular regulatory responses including the heart rate and peripheral resistance baroreflexes. The application of CSI involves the use of echocardiography for continuous beat to beat measurement of stroke volume. In the project led by Professor Williams, titled Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity, investigators characterize the renal and endocrine responses. Finally, in second project led by Professor Cohen, titled Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated Microgravity, investigators apply novel techniques to quantify changes in the cardiac conduction processes and assess any increased tendency toward cardiac arrhythmias or cardiac electrical alterations. Together, these projects cover a broad spectrum of systems involved in maintaining cardiovascular homeostasis and promise to provide new insight regarding their alterations in response to the major environmental changes of microgravity and disruption of circadian rhythms. The data from these studies is being used to develop and test potential countermeasures so as to ensure the health, productivity, and safety of astronauts during and on return from extended missions (*e.g.*, those that will occur on the International Space Station).

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I. PROJECT RESEARCH ACTIVITY

The Core provides the infrastructure for the human studies experimental protocols. The data derived from these protocols will be used to test specific hypotheses (detailed in the specific project proposals) regarding the effects on simulated space flight on cardiovascular regulation and function, vasoreactivity, hormones that affect the cardiovascular system and/or modify renal function, and cardiac electrical stability. There are two overriding hypotheses for the human studies project:

- Simulated microgravity will interfere with the cardiac, renal, and cardio-endocrine systems basally and in response to acute stress.
- Simulated microgravity and sleep deprivation will have additive effects on the responsiveness and regulation of cardiovascular homeostatic systems.

Specific Aim:

The concept of the Core resides in the realization that there are many factors involved in cardiovascular regulation. Each factor can only be interpreted if the other factors are tightly-controlled. For example, each of the factors to be addressed in this proposal can be modified by noise, time of day, level of stress, levels of sodium and potassium, caloric intake, time of food intake, sleep, temperature, humidity, and other environmental factors. On a space station, many of these factors are controlled differently than on Earth. Yet, the potential exists that a micro-environment can be created in space which theoretically can be structured to maintain cardiovascular integrity. To accomplish this, reliable data are required. Interpretation of ground-based data and previous studies in space related to cardiovascular deconditioning and fitness are variable and inconsistent. This is likely due to the lack of control of many of the variables noted above, particularly dietary intake. As in any experimental paradigm, to develop effective countermeasures, one needs to understand the mechanisms leading to the dysfunctional results. The aim of the Human Studies Core is to provide the infrostructure by which the three individual projects, "Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated microgravity", "Renal and Cardioendocrine responses in Humans to Simulated Microgravity" and "Alterations in Cardiovascular Regulation and Functuion during Simulated Microgravity", can achive this end (The spesific goal of each of this projects is contained within their Final Project Reports).

Overall Design

Each of the two experimental protocols consists of four phases. Phase 1 involves implementing a controlled diet for three days in an inpatient setting. Phase 2 begins a set of basal measurements and measurements during the application of acute stressors made in a rigidly controlled environment. In Phase 3, the chronic environmental change, simulating the environment of space travel, is imposed. Finally, in Phase 4, the set of basal and acute stressor studies is repeated.

Facilities

General Clinical Research Center (GCRC) – Brigham and Women's Hospital. The GCRC of the Brigham and Women's Hospital was established in 1961. It provides extensive support for inpatient and outpatient studies and statistical, laboratory, and dietary components of patient-oriented and applied research projects. The current five year budget, which was activated December 1, 1994, is $22 million. The inpatient component is located on the ninth floor patient tower of the Brigham and Women's Hospital. All beds in the newly-renovated GCRC (five IPM and eight other GCRC beds) are wired for continuous EEG, ECG, and temperature recordings using either Alice or Ultrasom systems. Its Core Laboratory (~1,800 sq. ft.) performs a wide range of hormonal assays. The Ambulatory General Clinical Research Center (~4,000 sq. Ft.) has five beds and utilizes a full-time research nurse. Other facilities on the GCRC include a clinical centrifuge, a cold room for special handling of blood following collection, and two metabolic kitchens for preparation of required measured diets. The Scientific Advisory Committee of the GCRC reviews proposals and makes recommendations to investigators and trainees on study design. The highly skilled nursing staff is associated with both the inpatient and outpatient components of the GCRC and performs a wide spectrum of techniques in clinical investigation.

Ambulatory General Clinical Center. The Ambulatory GCRC, located in more than 3,000 sq. ft. of space, consists of a waiting room, ten examination rooms, a nurses' office, two infusion rooms, a procedure room, a metabolic kitchen, a patient dining area, a conference room, and a small laboratory/sample processing area. A research nurse and secretary during regular business hours staff the Ambulatory GCRC. This facility is used to assist our research staff in screening procedures, which include physical examinations, electrocardiograms (ECGs), and the administration of additional tests required in the selection of subjects for the inpatient protocol. It is also available for any post-hospitalization studies that may be required.

Core Laboratory. The Core Laboratory of the GCRC was established in 1961 and currently is the largest Core Laboratory supported by the National Institutes of Health GCRC program. Currently 40 analytes are measured in the Core Laboratory. GCRC investigators process more than 50,000 assays per year. An individual investigator cannot use more than 5% of the GCRC's Core Laboratory's budget. However, users with

a large number of assays still can draw on the expertise and cost savings of this facility by paying the prorated cost of those samples above this limit. In most circumstances, this is a contribution of personnel and supply costs from primary project budgets. In its extended capacity (with additional users of the GCRC and a physical merger with a Core Laboratory for a Specialized Center of Research in Hypertension (SCOR) program supported by NIH), more than 100,000 assays are processed per year.

Computerized Data-Based Management and Analysis Systems (CDMAS). The GCRC has one of the longest involvements with a CDMAS resource. The GCRC also has been the leader in developing innovative uses for computer handling of large data bases. CDMAS personnel are responsible for the maintenance of the many pieces of electronic monitoring equipment in the IPM facility, data storage, and data transfer to investigators for appropriate analysis. Additionally, the GCRC provides support for a biostatistician.

Core Research Protocols

Protocol 1: Simulated Microgravity

Rationale: While there are no perfect ground-based models of weightlessness in a controlled environment where a variety of physiologic factors can be assessed simultaneously, the one that has been most commonly used is prolonged head down tilt bed-rest. Protocol 2, which is identical to Protocol 1, except that subjects sleep only 6 hours per night instead of 8 hours, will be initiated in the final quarter of Year 2.

Experimental Subjects: Healthy male, and post-menopausal female volunteers are recruited for study. Only subjects who have provided written, informed consent for their participation are considered. Those volunteers who meet all of the screening criteria are selected. Sample size estimates were derived from what is assumed to be the least sensitive and/or precise of the experimental techniques. Estimates were calculated assuming type I and II error probabilities of 0.05. Since both measurements during control and simulated space flight conditions are made for each subject, paired analysis is appropriate. From previous data, the procedure that was assumed to be the least sensitive to the microgravity was the plasma renin activity response to upright posture. Given the variability of the response characteristic in normal subjects on a high sodium diet and assuming that only a 20% change in the time response curve would be of physiologic significance, data from 12 patients would be required. Based on our preliminary experience with these types of studies, we anticipate about a 15% loss rate for individual data either because of incomplete studies, lost samples, or laboratory errors. Thus, 14 subjects are needed for this protocol.

Experimental Procedure: After completion of the screening procedures, subjects will begin the first of four phases of their study. Phase 1 lasts three days and takes place in the GCRC where they begin a constant, isocaloric, diet consisting of 200 mEq sodium, 100 mEq potassium, and 2500 mls fluid. This phase equilibrates the subjects in terms of caloric and electrolyte intake, since these factors can modify hormonal, renal, and cardiac responses. Throughout their in-patient course, the subjects are maintained on a constant light (16 hour)/dark (8 hour) cycle. They are not permitted to doze during their light cycle, which begins at 6:00 a.m. In addition, routine vital signs and daily weights are recorded every 8 hours. During Phase 2, the subjects continue the diet and 24 hour urine

samples are collected daily for the duration of the hospital stay. Total volume, creatinine, sodium, potassium, calcium, magnesium, chloride, phosphate, bicarbonate, and cortisol are measured daily with additional measurements at selected times (see Experimental Measurements). During Phase 2 of the protocol in the GCRC environment all of the measurements listed below in the section Experimental Measurements, as well as those listed in the section Acute Stressors, are made to establish pre-head down tilt bed-rest baseline values.

On completion of Phase 2, the subjects begin Phase 3 which is the maintenance of head down tilt bed-rest for sixteen days. During the final two days of head down tilt bed-rest all of the measurements listed below in the section Experimental Measurements are repeated.

Phase 4 is the recovery phase which lasts three days. During this phase, the subjects are allowed ad lib activity but must continue the constant diet. On the morning of the first and third days of Phase 4, the acute interventions are repeated. Thus, the impact of simulated microgravity on basal levels of the cardiac, vascular, renal, and endocrine systems are assessed as well as their responses to the acute interventions. Two control periods, one before and one after the simulated microgravity phase, are used.

Experimental Measurements

1) Cardiovascular Signal Monitoring for Cardiovascular System Identification. Four physiologic signals – ECG, instantaneous lung volume (ILV), blood pressure, and stroke volume – are recorded continuously and non-invasively for off-line CSI analysis. The ECG is recorded from the surface lead II. ILV is recorded using a two-belt chest-abdomen inductance plethysmograph (Respitrace system, Ambulatory Monitoring Systems, Inc.). An 800 cc inflatable bag serves as a calibration reference for ILV. Continuous blood pressure is recorded from the middle finger of the left or right hand using a fingertip cuff transducer (2300 Finapres BP monitor, Ohmeda, Inc.). Left ventricular stroke volume (SV) is recorded on a beat-to-beat basis using an ultrasound Doppler method [Eriksen & Walløe, 1990]. A bi-directional ultrasound Doppler velocimeter (CFM 750, Vingmed Sound, Horton, Norway) is operated in pulsed mode at 2 MHz with a hand-held transducer. The ultrasound beam is directed from the suprasternal notch toward the aortic root, and the sample volume is positioned centrally in the aorta some 1-2 cms above the aortic valve. Separately, the constant diameter of the rigid aortic ring is determined by parasternal section-sector-scanner imaging (CFM 750, Vingmed Sound, Horton, Norway). (Professor Cohen)

2) Standard Urine Collections. Twenty four hour urine assessment of creatinine, sodium, potassium, calcium, magnesium, chloride, phosphate, and cortisol are obtained on each day of the study. (Professor Williams)

3) Plasma Volume. Plasma volume is measured by a dilution technique using Evans Blue Dye, a non-toxic, non-carcinogenic, non-radioactive dye which binds to serum albumin. A pre-dye blood sample is drawn, then 10 ml of Evans Blue dye (1 mg/ml) is injected into an intravenous catheter. Five minutes later, another blood sample is drawn. Spectrophotometry at 615 nm wavelength is performed on the plasma fraction of this

sample to determine plasma volume. This measure is performed before and after the period of head down tilt bed-rest. (Professors Cohen and Williams)

4) Renal Blood Flow. Measuring para-aminohippurate (PAH) clearance assesses renal blood flow. A PAH loading dose (12 mg/kg) is administered acutely, and then a continuous infusion of PAH is maintained for the duration of the study. After a 60 minute equilibration period, three blood samples are obtained five minutes apart for PAH level determination. PAH levels are measured using an automated system. Glomerular filtration rates (GFRs) are measured by the creatinine clearance technique. (Professor Williams)

5) Urine Cortisol and Catecholamines. On the last days of the control and perturbation phases and the first and second days of the recovery phases, urine cortisol and catecholamines are measured. (Professor Williams)

6) Plasma Renin Activity, Aldosterone, Cortisol, Atrial Natriuretic Peptide, Vasopressin, Catecholamines, sodium, potassium, calcium, magnesium, chloride, and phosphate. These hormones are measured by standard techniques within the Core Laboratory on the last days of the control and perturbation phases and the first and second days of the recovery phases. (Professor Williams)

7) Angiotensin Infusion Test. Angiotensin II amide (Ciba, Summit, N.J.) is infused at rates of three and six ng/kg/min for 30 minutes after a 60 minute control period. A non-invasive Dinamap device records blood pressure and pulse every two minutes. Aldosterone levels are obtained at the end of the control period and each dose periods. (Professor Williams)

8) Norepinephrine Infusion Test. Norepinephrine is infused at rates of 30, and 60 ng/kg/min for fifteen minutes at each dose. Blood pressure and pulse are recorded every two minutes using a non-invasive Dinamap device. (Professor Williams)

9) Measurement of Microvolt Level T Wave Alternans. ECG data is collected during the upright bicycle exercise portion of this study for the analysis of microvolt level T wave alternans – a sensitive predictor of susceptibility to ventricular arrhythmias. (Professor Cohen)

10) 24 Hour Holter Monitoring. ECG data is collected with a 24 hour Holter monitor in order to detect potential ventricular ectopic activity and to quantify susceptibility to ventricular arrhythmias in terms of long term heart rate variability measurements. (Professor Cohen)

11) Echocardiography. Pre and post bed-rest echocardiograms are performed in all subjects following the acute stressor interventions. Two dimensional echocardiograms are performed in standard parasternal long, short axis, apical four and two chamber views. Real time cardiac volume and beat-to-beat ejection fraction are obtained at end expiration (average of five consecutive beats). Border detection algorithms are used to enhance endocardial tracking for accurate and reproducible measurements. Frame-by-frame digital display of echocardiographic volumes and ejection fraction are available for input into a PC platform for correlation with autonomic indices as measured by the CSI system. Left ventricular inflow measurements at the mitral leaflet tips are performed

from the apical four chamber views for analysis of indices of diastolic function including the E-wave, the A wave, E/Z ratio, deceleration time and isovolumic relaxation time. Digital storage retrieval is done utilizing removeable and rewriteable optical disks All Echocardiographic studies are performed by Dr Ofili or by a GCRC Cardiovascular Technician trained by Dr. Ofili. All data is forwarded to Dr. Ofili on a weekly basis for critical review and off-line analysis at the Morehouse School of Medicine. Analyses are performed using a Tomtec digital image analysis system. (Drs. Elizabeth Ofili and James Thomas)

12) Leg Compliance Study. Venous occlusion plethysmography with multiple proximal occlusion pressures is used to obtain compliance measurements. (Professor Cohen)

13) Right Atrial Transmural Filling Pressure. Central venous pressure is measured via a fluid filled catheter inserted via an antecubital vein, and esophageal pressure is measured via an air filled balloon catheter. These measurements are made during the acute stressor tilt table test performed during the supine to upright posture study as described below in the Acute Stressors section.

14) Countermeasure evaluation. At 09:00 the morning of the final day of the bed-rest period each subject is randomized to receive either 5 mg midodrine, an oral alpha-agonist vasoconstrictor drug, or placebo. Both subjects and study staff are blind to what is received. At 10:00 the Supine to Upright Posture Study (described below) takes place. At intervals throughout this study, all physiologic signals for CSI, Right Atrial Transmural Filling Pressure, and Leg Compliance are measured (described above). These measurements, in conjunction with recording the time until first pre-syncopal symptoms are experienced by the subject, provide the basis for evaluating the midodrine countermeasure.

Acute Stressors

1) Supine to Upright Posture Study. Subjects are woken up at 06:00 and remain supine until 10:00. At 120, 10, and 2 minutes prior to the beginning of upright tilt, blood samples are obtained. At 10:00, the subject is tilted upright on a tilt table to thirty degrees for ten minutes. The angle of tilt is then increased to sixty degrees for ten minutes. Finally the subject stands upright quietly with additional blood samples obtained from an indwelling catheter at repeated intervals until 12:40. Plasma renin activity, aldosterone, cortisol, norepinephrine, epinephrine, and dopamine are measured from these blood samples. Physiologic signals for CSI and right atrial transmural pressure are monitored at various points throughout this study. (Professors Cohen and Williams)

2) Upright Bicycle Exercise. Exercise is performed on a computer controlled bicycle ergometer. The exercise protocol involves graded increases in pedaling resistance until a heart rate of 70% of maximum predicted heart rate is achieved. Heart rate is sustained at or above a minimum rate of at least 105 beats per minute for at least 256 beats. Surface electrodes (CH 2000 multi-segmented electrode set) are used to record ECG signals for determination of microvolt level T wave alternans. Blood samples are drawn prior to

exercise, and at the end of the exercise for plasma renin activity, aldosterone, cortisol and catecholamines. (Professors Cohen and Williams)

The Human Studies Core has evaluated thirty subjects of which 23 have comleated the entire protocol. The experimental results are reported in the individual projects: "Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated microgravity", "Renal and Cardioendocrine responses in Humans to Simulated Microgravity" and "Alterations in Cardiovascular Regulation and Functuion during Simulated Microgravity".

IV. IMPLICATIONS FOR FUTURE RESEARCH

The overarching aim of our research is to gain a better understanding of pathophysiologic cardiovascular, renal and cardio-endocrine changes resulting from microgravity exposure so that effective countermeasures may be developed. While current research is mandated to be limited to ground-based studies, the near term focus is clearly on space-based studies. To this end we are using powerful minimally invasive and non-invasive techniques in these ground-based studies that can readily be adapted for in-flight use aboard the Space Shuttle or Space Station.

We did not study woman subjects under the current protocol because of cyclic hormonal changes, which would require specific modifications in protocol design. It has been observed that orthostatic intolerance following spaceflight is more pronounced among women than among men. To clarify this issue, we plan to study a population of menstruating females using the same protocol used in our previous study to test the hypotheses that females have the same patterns of renal sodium handling in response to microgravity as do men, but have less orthostatic tolerance.

Study results demonstrated age related differense in orthostatic tolerance following exposure to microgravity and in augmentation of PRA and aldosterone levels in response to upright posture following bed rest. In addition, older subjects tended to retain sodium more aggressively in response to bed rest. This difference may be a function of differential renal sodium handling, or of age, or of both. To clarify this issue we plan to study a population of men over 50 years of age using the same protocol used in our previous study. These data will be compared with the data set generated in predominantly younger men (<35 years of age) to test the hypothesis that age will modify the response pattern of the RAAS following microgravity exposure and that these modifications correlate with orthostatic tolerance. We also plan to test the effectiveness of spironolactone at preventing the development of changes in human myocardial electrical conduction properties following exposure to simulated microgravity in this subject group.

Cardiovascular system identification is a powerful methodology for assessing non-invasively closed-loop hemodynamic regulation. T-wave alternans analysis is a powerful methodology for assessing non-invasively changes in cardiac electrical stability. All of the renal and cardio-endocrine measurements made in this study rely simply on blood sampling, and are well within the capabilities of current space crews. Therefore, in future years virtually this entire Human Studies Core protocol

can be conducted in actual microgravity conditions, and the methodologies used here can be further used to test the effectiveness of potential countermeasures during actual spaceflight

Appendixes:

A. See Final Project Reports "Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated microgravity", "Renal and Cardioendocrine responses in Humans to Simulated Microgravity" and "Alterations in Cardiovascular Regulation and Functuion during Simulated Microgravity"

B. None

C. 3 papers submitted to JAP

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

Project Title: *Alterations in Cardiovascular Regulation and Function during Simulated Microgravity*

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Date

EXECUTIVE SUMMARY

Alterations in cardiovascular regulation and function that occur during and after space flight have been reported. These alterations are manifested, for example, by reduced orthostatic tolerance upon reentry to the earth's gravity from space. However, the precise physiologic mechanisms responsible for these alterations remain to be fully elucidated. Perhaps, as a result, effective countermeasures have yet to be developed. In this project we applied a powerful, new method – cardiovascular system identification (CSI) – for the study of the effects of space flight on the cardiovascular system in order to develop effective countermeasures.

CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate, arterial blood pressure (ABP), and instantaneous lung volume (ILV – respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of all the physiologic mechanisms coupling these signals, CSI provides a model of the closed-loop cardiovascular regulatory state in an individual subject. The model includes quantitative descriptions of the heart rate baroreflex, autonomic function, as well as other important physiologic mechanisms.

We applied CSI in conjunction with the bed rest protocol of the Human Studies Core project. This protocol involves ground-based, human head down tilt bed rest to simulate microgravity and acute stressors – upright tilt, standing and bicycle exercise – to provide orthostatic and exercise challenges. We found that a number of autonomically mediated responses, in particular the heart rate baroreflex, were diminished as a result of head down tilt bed rest.

Based on review of our preliminary human data, as well as based on animal data and computer simulation data obtained by other investigators in the NSBRI Cardiovascular Alterations Team, we decided to test a pharmacologic countermeasure which is applied at the very end of the bed rest period. This countermeasure was applied using the same bed rest protocol used to obtain the control data. This double blinded prospective evaluation of the countermeasure demonstrated that it was successful in diminishing orthostatic intolerance.

This project has led to better understanding of the mechanisms of orthostatic intolerance and the identification of a potential pharmacologic countermeasure. The CSI methodology used in this study can be also applied to the study of patients with a range of diseases that alter closed loop cardiovascular regulation such as heart failure, diabetes, and hypertension and may also be used to monitor treatment of these patients.

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PROJECT RESEARCH ACTIVITY

Background

Postflight Orthostatic Intolerance

The cardiovascular system undergoes significant alterations during spaceflight, which appear to serve well during flight. These changes include in-flight reductions in heart rate and arterial pressure (10) as well as changes in autonomic control of arterial pressure (11). However, these in-flight adaptations do not serve well upon return to Earth's gravity, when a significant number of astronauts suffer from orthostatic hypotension. About 20% of astronauts after short missions and 83% of astronauts after long missions are not able to support standing arterial pressure for ten minutes (1). In one study the incidence was as high as 67 % (12). Current countermeasures involve oral loading with salt and water and inflation of an anti-gravity suit prior to re-entry. However, these measures have not prevented the occurrence of orthostatic hypotension.

Post-flight orthostatic intolerance is thought to have several contributing factors, including reduced intravascular volume (13,14), and dysfunction of autonomic regulation of arterial pressure (11, 15-16), which can prevent compensatory responses to venous pooling during upright posture. A recent study showed that astronauts who suffer most severely from postflight orthostatic hypotension lack appropriate responses of the sympathetic nervous system, as evidenced by lower standing plasma norepinephrine levels, and lower standing total peripheral resistance, than astronauts who are less severely affected (16). In this same study, women were observed to have a higher incidence of post-flight orthostatic intolerance than men, however many of the men tested were also career high performance jet pilots, and have a higher degree of orthostatic tolerance than non-pilots. As most of the women were non-pilots, this effect is hard to distinguish from the gender effect.

Investigators have examined the role that alteration in specific reflexes may play in the development of orthostatic intolerance, but the precise etiology of post-flight orthostatic intolerance remains unclear. A wide-range of cardiovascular parameters have been studied. There is some evidence that reduced heart rate baroreflex responsiveness may play a role. In studies employing a neck chamber to stimulate carotid baroreceptors, the gain of the heart rate baroreflex was found to be reduced after prolonged head-down tilt bed rest (17), and after short duration (15) and longer duration spaceflight (11). Other studies have examined alterations in autonomic function after spaceflight that might contribute to the development of orthostatic intolerance. In one study, reductions in parasympathetic and increases in sympathetic activity, as determined from Valsalva responses, were reported after spaceflight (11). Spectral analysis of heart rate fluctuations suggest that parasympathetic activity remains unchanged after spaceflight, and that sympathetic activity is either increased or unchanged (11, 18).

Alterations in cardiac systolic and diastolic function may also represent a potential mechanism responsible for orthostatic intolerance. Magnetic resonance imaging on four members of the D-2 German Spacelab mission showed a significant loss of myocardial mass after a ten-day mission (19). Additionally, in a recent study, changes in left ventricular pressure-volume characteristics were found to develop over a two-week period of head-down tilt bed rest producing a stiffer ventricle with reduced end-diastolic volume (20). However, in another study, cardiac contractility, measured in terms of velocity of circumferential fiber shortening, did not change after spaceflight (21).

Data from Skylab-3 and -4 suggest leg blood flow and compliance increase during the early hours of spaceflight (22), and ground based studies simulating microgravity have documented an increase in leg vessel compliance (23-25). Such changes, in conjunction with the well-documented decrease in plasma volume in space (13,14), may contribute to orthostatic intolerance following microgravity exposure by decreasing venous return, and thereby cardiac stroke volume.

Cardiovascular System Identification

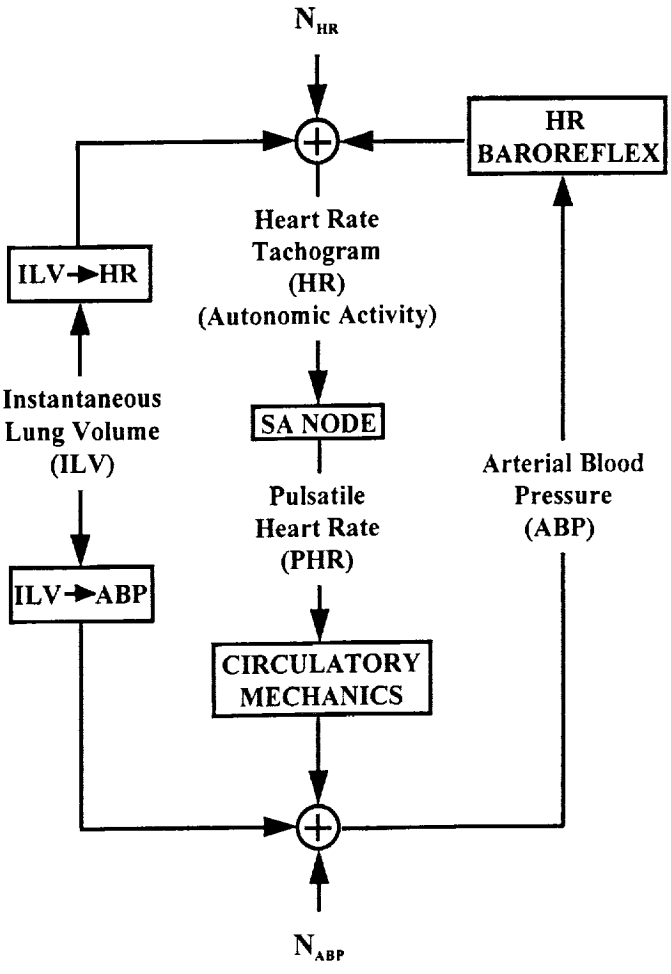
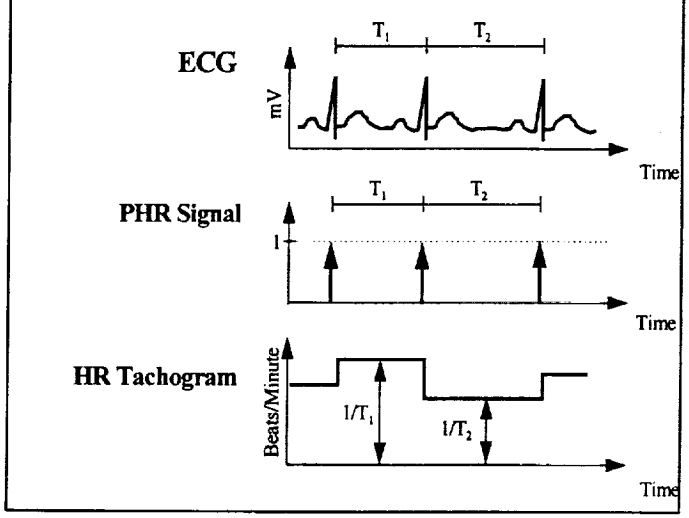


Figure 1: CSI model of short-term cardiovascular regulation relating HR, PHR, ABP, and ILV. See text for description (From reference 40).

We have developed techniques to study the feedforward and feedback couplings involved in closed-loop cardiovascular regulation based on parametric system identification techniques applied to spontaneous fluctuations in cardiovascular variables (39-44). We are applying these techniques, termed Cardiovascular System Identification (CSI), to investigate cardiovascular deconditioning associated with weightlessness.

CSI provides a novel, non-invasive means for the quantitative characterization of cardiovascular regulation and function in an individual subject. CSI quantifies physiologic coupling mechanisms by mathematically analyzing the relationship between

Figure 2: The relationship between HR, PHR, and ECG (From reference 40).



ongoing second-to-second fluctuations in non-invasively measured physiologic variables such as heart rate and ABP. In this way, important physiologic coupling mechanisms, such as the heart rate baroreflex and other measures of autonomic function, can be quantitatively described. By characterizing multiple coupling mechanisms, one can construct an individualized model of closed-loop cardiovascular regulation for each subject. The CSI approach may be adapted to the number of physiologic signals available for analysis. In general, if one measures *n* physiologic signals in a subject, *n(n-1)* couplings can be identified. Thus, as more signals are made available, more physiologic coupling mechanisms may be characterized resulting in a more detailed model of closed-loop cardiovascular regulation.

We have developed a CSI method to identify the couplings between second-to-second fluctuations in heart rate, ABP, and respiratory activity – in terms of time variations in ILV. The couplings between these variables that are identified are depicted in the closed-loop model of cardiovascular regulation in Figure 1. The model consists of five couplings — CIRCULATORY MECHANICS, HR BAROREFLEX, SA NODE, ILV→HR, and ILV→ABP — each of which represents a distinct physiologic mechanism.

CIRCULATORY MECHANICS represents the relationship between cardiac contraction and the generation of the ABP waveform. The input to CIRCULATORY MECHANICS is pulsatile heart rate (PHR) which is defined to be a train of impulses occurring at the times of contraction of the ventricles (Figure 2, middle trace). PHR may be constructed from the times of occurrence of the QRS complexes in the electrocardiogram (ECG). The output from CIRCULATORY MECHANICS is the pulsatile ABP signal. The CIRCULATORY MECHANICS transfer function represents the ABP wavelet generated with each cardiac contraction. CIRCULATORY MECHANICS is determined by the contractile properties of the heart as well as the mechanical properties of the great vessels and the peripheral circulation. CIRCULATORY MECHANICS may also encompass the reflex adjustment of vascular mechanical properties mediated by the α-sympathetic, β-sympathetic and renin-angiotensin systems (the resistance baroreflex).

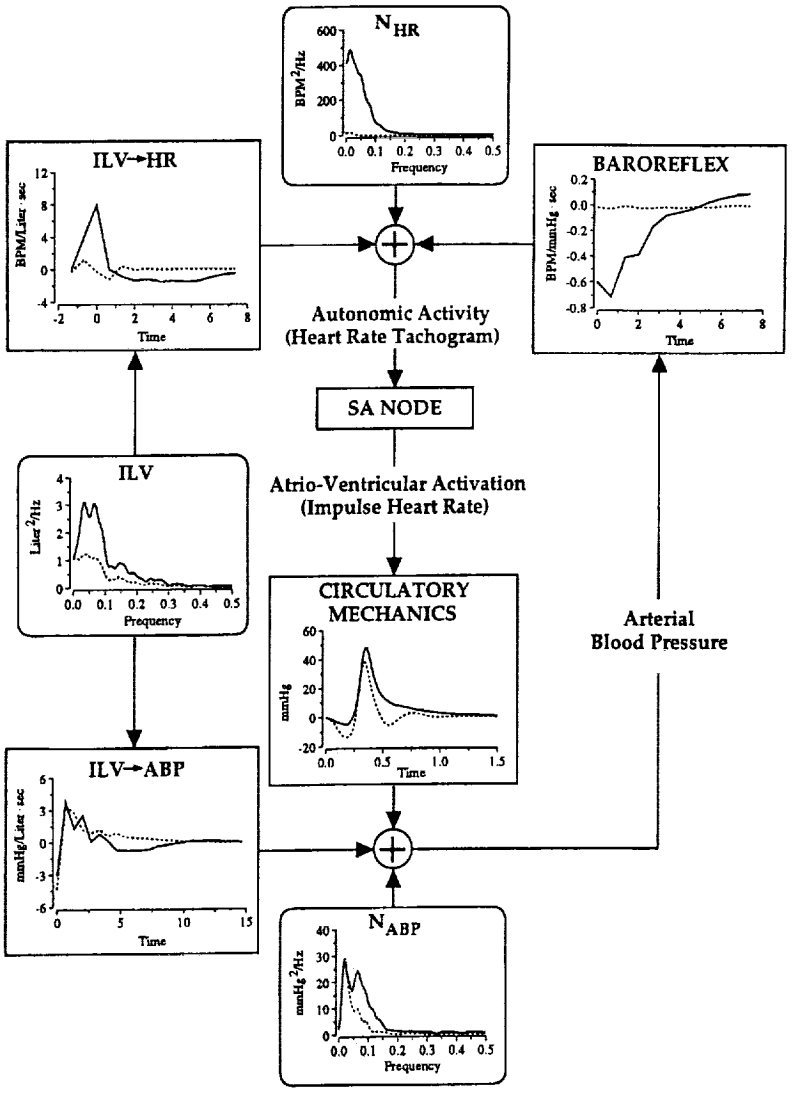
HR BAROREFLEX represents the autonomically mediated baroreflex coupling between fluctuations in ABP and fluctuations in heart rate. Here heart rate is represented by the heart rate tachogram (HR) rather than PHR. HR is defined to be a stepwise continuous process (Figure 2, bottom trace) whose value corresponds to the reciprocal of the current inter-beat interval for the time period corresponding to the duration of that interval (45). Unlike PHR, HR has no periodic component at the mean heart rate frequency and is closely related to the net autonomic input signal modulating sinoatrial node activity. SA NODE represents the coupling between HR and

PHR. The SA NODE in this model is an "integrate and fire" device; such a device precisely relates the input HR to the output PHR.

ILV→HR represents the autonomically mediated coupling between respiration and heart rate. ILV→HR is responsible for the respiratory sinus arrhythmia. ILV→ABP represents the mechanical effects of respiration on ABP due to the alterations in venous return and the filling of intrathoracic vessels and heart chambers associated with the changes in intrathoracic pressure.

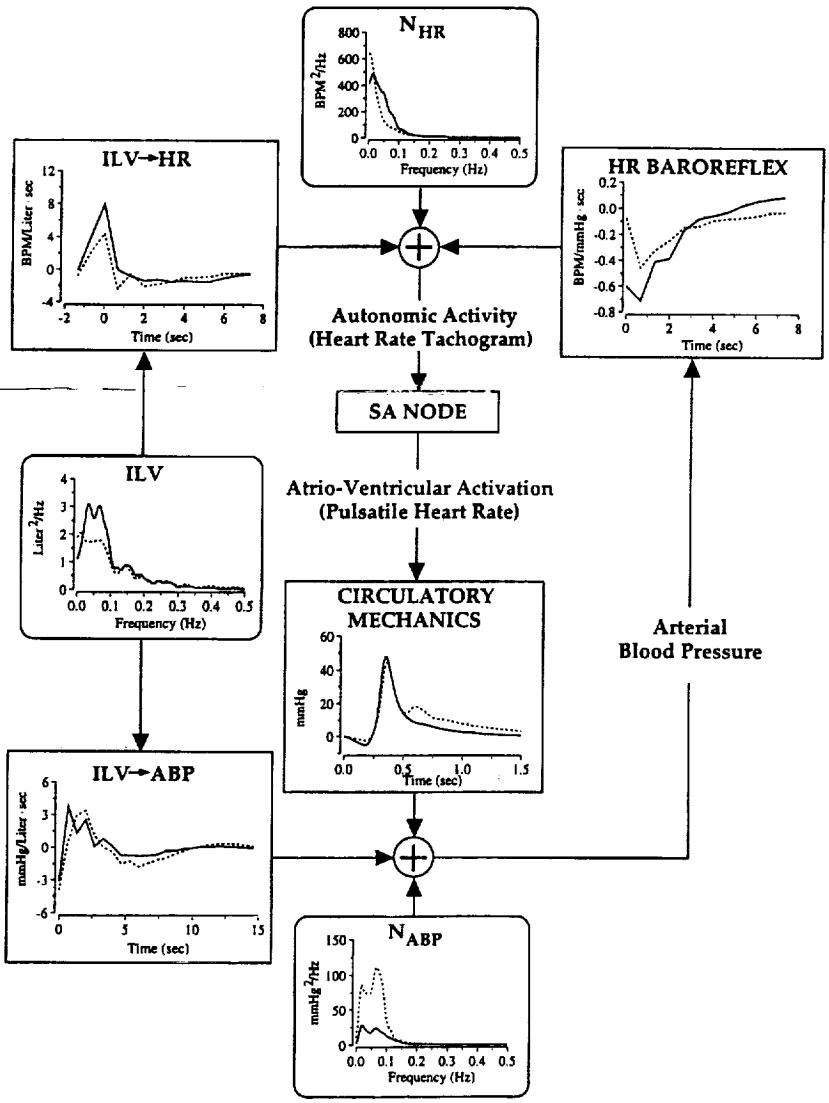
In addition to the five coupling mechanisms, the model incorporates two perturbing noise sources, N_{HR} and N_{ABP}. N_{HR} represents the fluctuations in HR not caused by fluctuations in ABP or ILV. Such fluctuations may result, for example, from autonomically mediated perturbations driven by cerebral activity. N_{ABP} represents fluctuations in ABP not caused by fluctuations in heart rate or ILV. Such blood pressure fluctuations may result, for example, from fluctuations in peripheral vascular resistance as tissue beds adjust local vascular resistance in order to match local blood flow to demand or from beat-to-beat fluctuations in stroke volume.

Figure 3: Group average CSI results for subjects in the supine posture before administration of any drugs [solid lines] and after parasympathetic and β-sympathetic autonomic blockade [dashed lines] (From reference 40).



The perturbations represented by N_{HR} and N_{ABP} as well as the variability in ILV are responsible for driving all fluctuations in HR and ABP through the coupling mechanisms. N_{HR} and N_{ABP} are not directly measured quantities. They represent the residual variability in HR and ABP once one subtracts out the components of variability caused by the fluctuations in each case

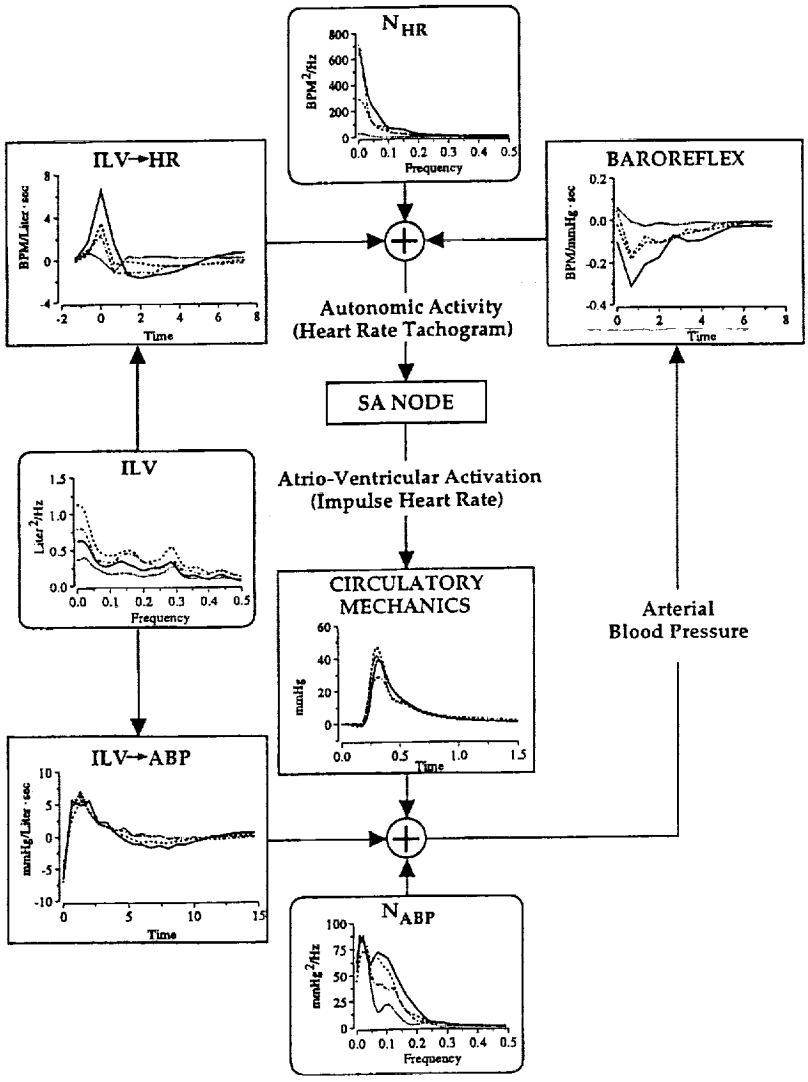
Figure 4: Group average CSI results for subjects in the supine posture [solid lines] and in the standing posture [dashed lines] (From reference 40).



in the other two measured signals. In order to completely identify the CSI model, six-minute segments of ECG, ILV measured using a non-invasive Respitrace system (Ambulatory Monitoring Systems, Inc.), and intra-arterial ABP are obtained. Data are collected during a random interval breathing protocol (46) during which subjects breathe in response to auditory cues at a comfortable mean rate of 12 breaths per minute but with inter-breath intervals randomly varying between one and 15 seconds. Subjects adjust their own tidal volumes thereby leaving blood gases unperturbed. The random interval breathing protocol broadens the frequency content of the recorded physiological signals, thereby facilitating CSI. Using this data, each coupling (except for SA NODE which is a predefined, nonlinear "integrate and fire" device) and perturbing noise source power spectrum may be identified from a pair of linear, time-invariant autoregressive moving average (ARMA) difference equations.

We evaluated CSI in 14 human subjects studied in the supine and standing postures under control conditions and under conditions of parasympathetic and β-sympathetic pharmacological blockade (40). Figure 3 above shows the group average CSI results of these subjects before (solid lines) and after (dashed lines) combined β-sympathetic and parasympathetic pharmacological blockade. Each coupling element is represented in the figure in terms of its

Figure 5: Group average CSI results for the three diabetic groups and the control group. Solid, dashed, dashed-dotted, and dotted lines respectively designate control, Minimal, Moderate, and Severe autonomic neuropathy groups (From 41).



estimated impulse response function – the response of the system to a transitory perturbation of arbitrarily short duration. The noise sources are represented in terms of their power spectra (energy as a function of frequency).

Note that during combined β-sympathetic and parasympathetic blockade, the amplitude of the autonomically mediated physiological coupling mechanisms (HR BAROREFLEX and ILV→HR) are both reduced essentially to zero as is the amplitude of the power spectrum of N_{HR} which represents autonomically mediated perturbations to heart rate. Conversely, the amplitude of the mechanically mediated couplings (ILV→ABP and CIRCULATORY MECHANICS) are preserved. There is a partial diminution in the power spectrum of N_{ABP}. The remaining power in N_{ABP} may reflect non-autonomically mediated perturbations to ABP resulting perhaps from local autoregulatory fluctuations in peripheral vascular resistance. There is also a reduction in amplitude of fluctuations in ILV representing decreased tidal volume perhaps due to β-sympathetic mediated broncho-constriction.

The change in posture from supine to standing results in a relative shift from parasympathetic toward sympathetic cardiovascular control as well as mechanical changes. Statistically significant changes occur in all coupling elements and the N_{ABP} power spectrum on a change in posture from supine to standing (Figure 4). However, the most substantial change observed is in the N_{ABP} power spectrum, which is markedly increased in the standing posture, presumably due to mechanical perturbations or changes in sympathetic modulation of local systemic resistance.

The results in Figures 3 and 4 demonstrate that CSI correctly predicts the effect of pharmacological blockade and postural changes. Our desire is to be able to apply CSI to the study of chronic changes in cardiovascular regulation which occur during and after space flight. As a ground-based model of chronic changes in autonomic function in man, we chose to study patients with diabetic autonomic neuropathy. Diabetic autonomic neuropathy represents a progressive deterioration in sympathetic and parasympathetic nervous system function in patients with diabetes (47). Diabetic autonomic neuropathy is related to the inadequacy of glucose control, and noninvasive monitoring of this condition is important as a physiological guide to the management of diabetes. We studied 60 diabetic subjects and 37 control subjects (41). The diabetic subjects were divided into three groups with Minimal, Moderate, and Severe autonomic neuropathy on the basis of standard autonomic testing. The Minimal group was indistinguishable from the control group on the basis of the conventional testing. CSI was performed totally non-invasively in these subjects with ABP recorded using a Finapres non-invasive transducer (Ohmeda, Inc.). Figure 5 shows that the CSI results, corrected for patient age, reveal a progressive diminution in the autonomically mediated physiological coupling mechanisms with increasing degree of autonomic neuropathy across the four groups, while the mechanically mediated couplings are not affected. Interestingly, the CSI results reveal a statistically significant difference in autonomic function between the control and Minimal groups which was not revealed by standard autonomic testing. The results of this study demonstrate, in this ground-based model, that progressive changes in autonomic function could be quantitatively assessed by CSI methods.

Midodrine

There is evidence that both venous return and peripheral vascular resistance are reduced after spaceflight. Though not the only contributors, both of these factors most certainly increase the incidence of post-spaceflight orthostatic hypotension and presyncope. Several studies have demonstrated a reduction in cardiac stroke volume upon return from space (12, 16, 29), and others have shown reduced resistance responses to standing, particularly in those astronauts who have the most difficulty maintaining arterial blood pressure while standing (12, 16). Midodrine is an agonist at α-adrenergic receptors located on smooth muscle in both veins and arterioles, and thus both reduces venous pooling and increases peripheral vascular resistance (30-32).

In our previous studies, following head-down bed rest some subjects exhibited a hemodynamic pattern consistent with vasovagal syncope during orthostatic stress. This is likely mediated through the Bezold-Jarisch reflex, which is thought to play a major role in vasovagal syncope (33, 34), and has been suggested as a possible mechanism for post-flight orthostatic intolerance (35, 36). Other subjects displayed a pattern of presyncope more consistent with dysautonomia (37,38), i.e., a steady decrease in blood pressure without a precipitous drop in heart rate. Importantly, both types of presyncope were alleviated with the use of midodrine.

Both of these patterns of presyncope seen in our bed rest study have been documented after spaceflight (12, 16). The occurrence of the Bezold-Jarisch reflex postflight may be provoked by hypovolemia and decreased venous return, which leads to ventricular contraction around a relatively empty ventricular chamber, stimulating inhibitory mechanoreceptors in the inferoposterior ventricular wall. The other pattern of postflight presyncope is associated with inadequate norepinephrine release and resistance responses (16). We suggest that the use of midodrine can alleviate both types of hypotension and presyncope following microgravity

exposure. We have now shown midodrine to be an effective countermeasure against orthostatic intolerance following simulated microgravity exposure in men (9), and now propose to study it in women, who are thought to be more susceptible to the problem than are men.

Experimental Results

Application of Cardiovascular System Identification techniques to head-down tilt bed rest subjects.

Fifteen male subjects in excellent health and with anthropometric characteristics similar to those of American astronauts [age = 33.5 +/- 11.3 (SD) years, height = 70 +/- 2.4 (SD) inches, weight = 76.8 +/- 7.6 (SD) kilograms] were selected after screening physical and psychological examinations. Screening laboratories and tests included a 12-lead electrocardiogram, complete blood count with differential, chemistry profile, thyroid function tests and urinalysis. The exclusion criteria included history or evidence for psychiatric disorders, hypertension, diabetes, coronary artery disease, renal insufficiency, thyroid disease, alcohol or drug abuse, viral hepatitis or anemia. The Brigham and Women's Hospital (Boston, Massachusetts) Research Committee approved the protocol and informed consent was obtained.

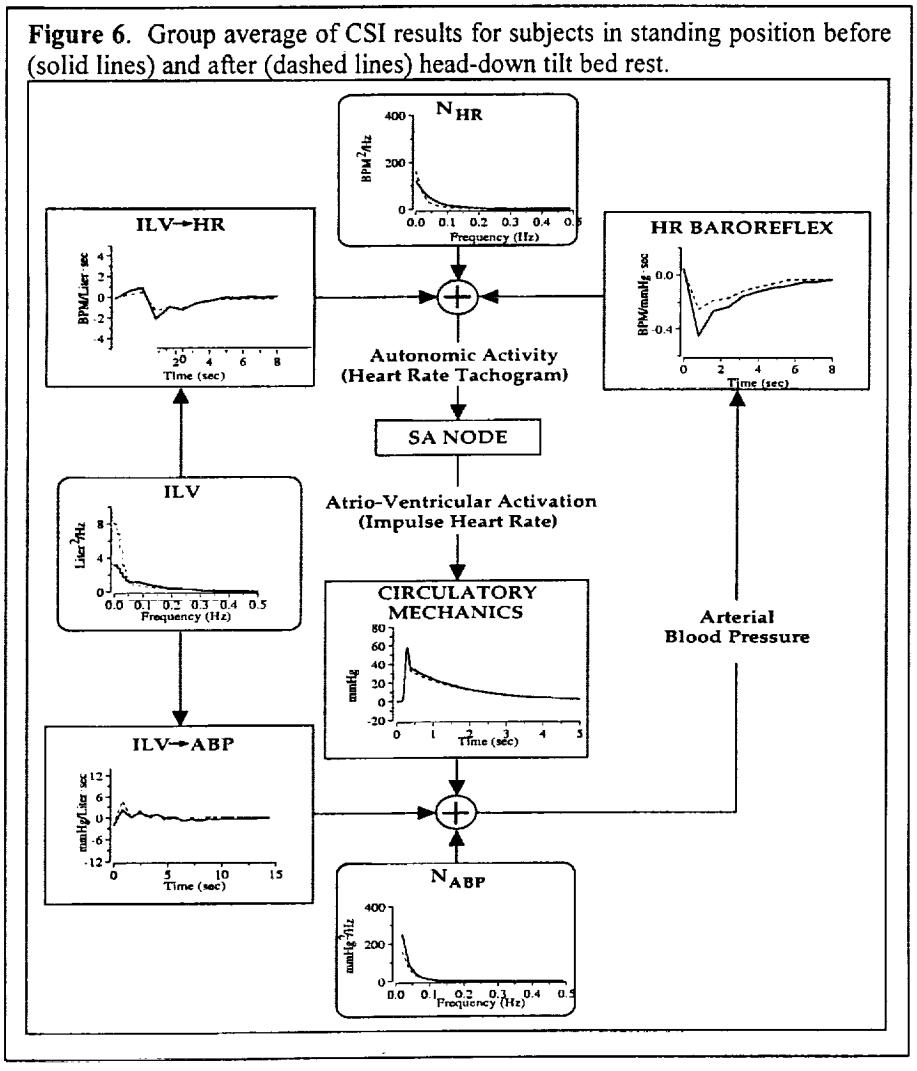


Figure 6. Group average of CSI results for subjects in standing position before (solid lines) and after (dashed lines) head-down tilt bed rest.

Experimental Protocol. Following the screening procedures, subjects were admitted to the Brigham and Women's Hospital General Clinical Research Center. They spent three (subjects 1-4) or five (subjects 5-15) days undergoing baseline testing and equilibrating to an isocaloric diet consisting of 200 mEq sodium, 100 mEq potassium, and 2500 ml fluid.

Subjects were then instrumented for CSI in conjunction with a tilt-stand protocol. Data acquisition for CSI involves non-invasively measuring and recording the surface electrocardiogram (ECG), arterial blood

pressure (ABP), and instantaneous lung volume (ILV), for each subject using an on-line dedicated data sampling and analysis program. The ABP signal is measured from the middle finger of the left or right hand using a Finapres (Ohmeda, Inc.) or Portapres (TNO, Netherlands) blood pressure monitor. The ILV signal is measured with a Respitrace (Ambulatory Monitoring Systems, Inc.) system two belt chest-abdomen inductance plethysmograph and calibrated with an 800 cc inflatable spirobag. For each CSI data acquisition period, we recorded these signals for 8 minutes. For data acquisition, subjects breathe according to a random interval breathing protocol (2) which requires them to breathe in response to auditory cues at a comfortable mean rate of 12 breaths per minute, but with inter-breath intervals randomly varying between one and 15 seconds. Subjects adjust their own tidal volumes thereby leaving blood gases unperturbed. The random interval breathing protocol broadens the frequency content of the recorded physiological signals, thereby facilitating CSI. Data for CSI was acquired with subjects laying supine on a tilt table, then at thirty degrees head up tilt, then at sixty degrees of head up tilt, and finally standing. Following these CSI data acquisition sessions, the subjects stood upright quietly for an additional 140 minutes for hormonal measurements for a parallel study. The test was immediately terminated if a subject experienced a sudden precipitous drop in blood pressure and had difficulty appropriately responding to questions, i.e., manifested mental status changes consistent with presyncopal symptoms.

Subjects then underwent -5° head-down tilt bed rest for nine (subject 1), 14 (subjects 2-4), or 16 days (subjects 5-15). Subjects were strictly confined to bed for the entire bed rest period. They ate all meals lying on their side, propped up with one elbow. They used a bedpan to urinate or defecate. The tilt-stand test with CSI data acquisition described above was repeated at the end of the bed rest period and again after two (subjects 1-4) or three (subjects 5-15) days of normal ambulatory activity. The data was then transferred to the Massachusetts Institute of Technology (M.I.T.) for subsequent data processing and CSI analysis.

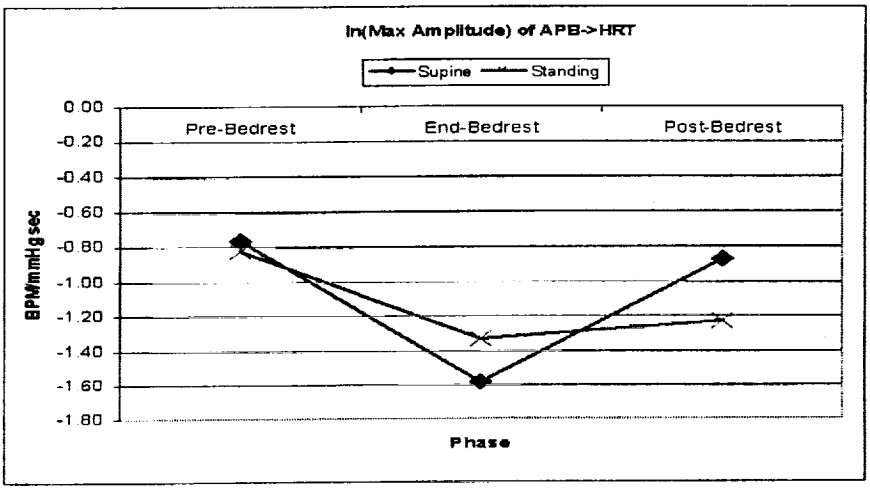
Table 1. Comparison of CSI parameterization for pre-bedrest vs. end of bedrest (i.e. within an hour of getting up from bed rest) vs. post-bedrest (i.e., two or three days after bed rest) for both supine and standing postures (mean ± standard error). An * denotes a parameter with a p-value < 0.05 with respect to the corresponding pre-bedrest parameter. An ^ denotes a parameter with a p-value < 0.05 with respect to the corresponding supine parameter.

Impulse Response	Condition	ln(Peak Amplitude)		Area		ln(Absolute Area)		ln(Characteristic Time)	
		Supine	Standing	Supine	Standing	Supine	Standing	Supine	Standing
ILV->HR	Pre-Bedrest	1.37±0.15	0.42±0.27^	-0.22±3.06	-4.71±3.03	2.53±0.16	2.58±0.15	1.03±0.14	1.40±0.17
	End Bedrest	1.14±0.16	0.30±0.26^	2.65±2.50	-3.46±2.76^	2.54±0.17	2.43±0.12	1.25±0.15	1.65±0.18
	Post-Bedrest	1.26±0.17	0.52±0.27^	6.35±4.72*	-3.47±4.64	2.76±0.15	2.71±0.16	1.04±0.20	1.61±0.19^
HR baroreflex	Pre-Bedrest	-0.76±0.13	-0.82±0.10	-0.72±0.12	-1.39±0.23^	0.08±0.11	0.47±0.10^	1.27±0.10	1.63±0.13^
	End Bedrest	-1.58±0.21*	-1.33±0.15*	-0.39±0.08*	-1.05±0.15^	-0.31±0.16*	0.13±0.13^	1.42±0.12	1.72±0.13
	Post-Bedrest	-0.87±0.08	-1.23±0.07*^	-0.81±0.25	-1.07±0.20	0.21±0.13	0.31±0.11	1.47±0.14	1.82±0.15
ILV->ABP	Pre-Bedrest	0.77±0.11	1.26±0.22^	-0.84±2.66	0.60±2.95	2.64±0.13	2.86±0.19	2.11±0.10	1.93±0.08
	End Bedrest	1.40±0.17*	1.00±0.24	5.90±4.02	4.17±4.02^	2.93±0.16	2.77±0.20	2.01±0.14	1.91±0.16
	Post-Bedrest	1.42±0.13*	0.94±0.26	2.89±4.71	-2.62±3.97	2.96±0.19	2.88±0.15	1.89±0.12	1.94±0.13
Circulatory	Pre-Bedrest	4.12±0.03	3.87±0.06^	67.25±2.76	55.48±3.26^	4.20±0.04	3.99±0.06^	0.57±0.04	0.67±0.06
Mechanics	End Bedrest	4.04±0.04*	3.62±0.07*^	61.21±3.66	45.59±3.02*^	4.08±0.07	3.79±0.07*^	0.54±0.05	0.67±0.09
	Post-Bedrest	4.13±0.04	3.76±0.05^	62.94±2.44	45.92±2.59*^	4.13±0.04	3.81±0.06*^	0.55±0.04	0.65±0.08

Results. Graphical representations of CSI results are presented for a subject in the standing (Figure 6) position before and at the end of simulated microgravity. Table 1 presents the data in numerical form. Each coupling element is represented in the figure in terms of its estimated impulse response function — the response of the system to a transitory perturbation of arbitrarily short duration. The noise sources are represented in terms of their power spectra (energy as a function of frequency). Note that following simulated microgravity exposure, the amplitude of the autonomically mediated HR BAROREFLEX is greatly reduced in both the supine and standing postures. The amplitude of the other autonomically-mediated coupling mechanism, ILV→HR, however, is not significantly changed by bed rest. The amplitudes of the mechanically mediated couplings (ILV→ABP and CIRCULATORY MECHANICS) show statistically significant changes following bed rest as well. In the case of ILV→ABP the mechanical influence of lung volume on arterial blood pressure is more pronounced following bed rest, and in the case of CIRCULATORY MECHANICS the pulse pressure is narrowed. Both of these latter mechanical effects are attributable to the well-documented decrease in circulating blood volume following actual and simulated weightlessness (13, 14). The decrease in amplitude of the HR BAROREFLEX indicates a defect of autonomic cardiovascular control mechanisms following simulated microgravity, which may be responsible in part for orthostatic intolerance. Figure 7 illustrates the decrease in peak amplitude of the HR BAROREFLEX, and shows that it does not completely recover to baseline within 48 to 72 hours of normal ambulatory activity. Table 1 above compares all CSI parameters (mean ± standard error) of the impulse response functions for pre-bed rest vs. end of bedrest (i.e., within an hour of completing the bed rest period) vs. post-bed rest (i.e., 2 or 3 days following bed rest) for both supine and standing postures. An * denotes a parameter with a p-value < 0.05 with respect to the corresponding pre-bedrest parameter. An ^ denotes a parameter with a p-value < 0.05 with respect to the corresponding supine parameter. While all parameters are presented, the Peak Amplitude parameter proved to be the most robust for comparing differences before and after bed rest.

Thus, the head-down tilt bed rest model of weightlessness causes a diminution in the gain of an important cardiovascular regulatory mechanism and affects to a lesser degree mechanical coupling mechanisms consistent with a decrease in circulating blood volume. These findings may help explain why the use of fluid loading alone prior to return to gravitational stress has not been entirely effective as a countermeasure against orthostatic intolerance following spaceflight. These findings suggest that fully effective countermeasures will either maintain the integrity of cardiovascular regulatory mechanisms while in flight, or augment or substitute for their diminished effectiveness upon return to gravitational

Figure 7. Peak Amplitude of HR BAROREFLEX before bedrest, on the last day of bedrest, and after 48 to 72 hours of ambulatory recovery from bed rest.

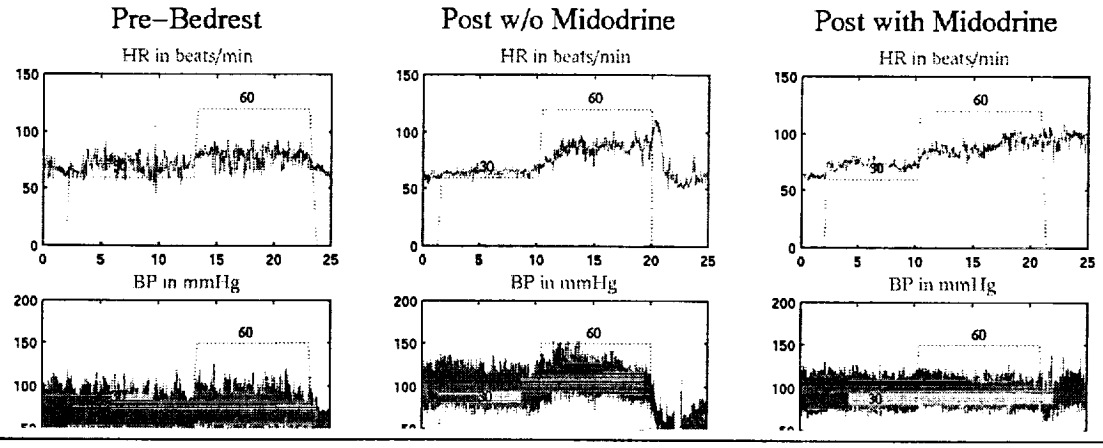


stress. We anticipate that future improvements in the CSI methodology will provide for the quantitative characterization of control of peripheral resistance. This will allow a more complete closed loop assessment of cardiovascular regulation.

Testing the effectiveness of midodrine at preventing orthostatic intolerance following exposure to simulated microgravity.

Experimental Protocol. From the same subject population described above for Specific Aim 1, ten subjects were randomized to receive midodrine (5 mg po) or placebo on a double blind basis on the final day of bed rest, one hour before the tilt-stand test. The ratio of midodrine to placebo was adjusted such that six of these ten subjects received midodrine. In addition, one of the original four subjects to complete the bed rest study without any countermeasure was re-studied several months later with the administration of 5 mg of midodrine in an open label design one hour before the post-bed rest tilt-stand test. Thus, eight untreated bed rest subjects are compared with seven subjects who received midodrine, one of which was in an open label design. All subjects who received midodrine underwent the longer 16 day period of head-down tilt bed rest,

Figure 8. Hemodynamic data from a normal human volunteer during a tilt test before bed-rest (left), following 14 days of head-down tilt bed rest untreated (center), and following 16 days of head-down tilt bed rest treated with midodrine one hour prior to upright tilt (right). These data are from the same subject studied on two separate occasions several months apart. The dashed lines indicate the periods of tilt to 30° and 60° of head up tilt. In the middle column the dashed lines at the end of the 60° head up tilt period indicate the pre-syncopal episode, at which time the subject was returned to a horizontal position.

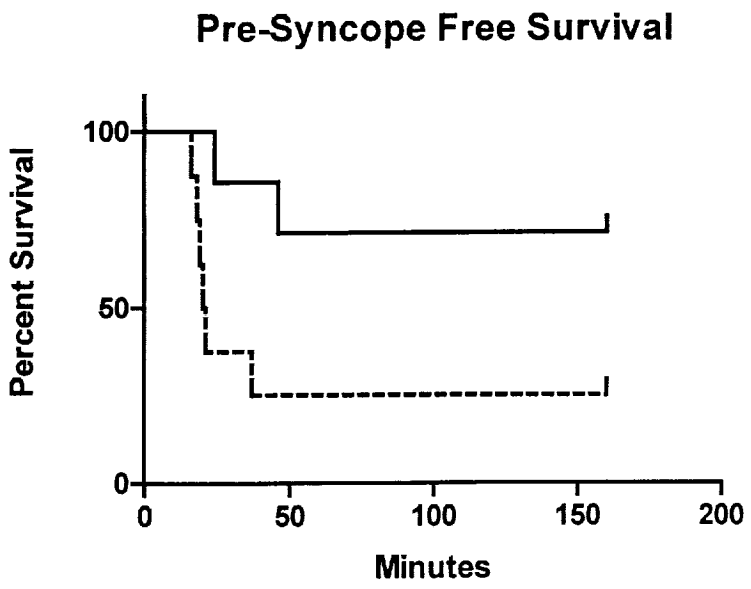


whereas four of eight control subjects underwent a somewhat shorter period of bed rest (nine days for the first subject, and 14 days for the second, third, and fourth subjects). The study protocol was the same in all subjects except for the shorter period of bed rest in the first four control subjects.

Results. The cardiovascular responses of one subject during three different tilt-stand tests are illustrated above in Figure 8. These data are from the only subject who was studied before and after two separate bed-rest studies, once with midodrine and once without midodrine. The column on the left illustrates the pre-bed rest tilt test. The center column illustrates the immediate post-bed rest tilt test without midodrine. The right hand column illustrates the immediate post-bed rest tilt test when this subject was treated with midodrine 5 mg one hour

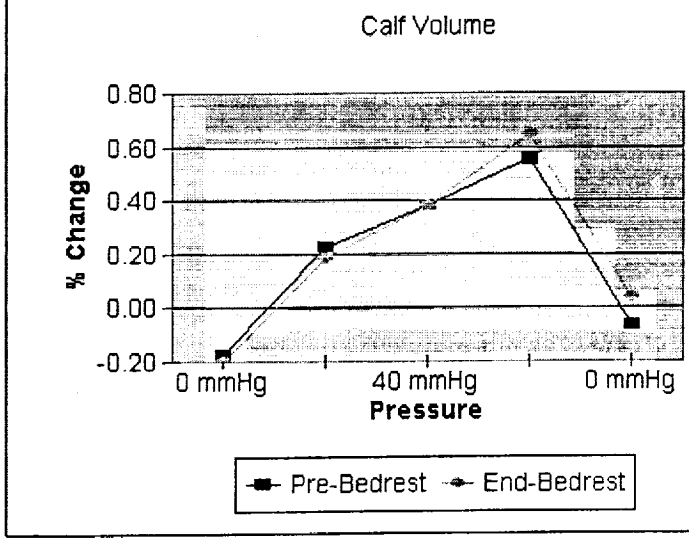
prior to upright tilt. The dashed lines separate the horizontal, 30 degrees upright, and 60 degrees upright tilt periods. In the middle column, the dashed lines at the end of the 60-degree tilt period correspond to the presyncopal event and the return of the subject back to the horizontal position. Note that in the post-bed rest without midodrine (middle column), arterial pressure began to trend down sharply just prior to 20 minutes, while HR continued to increase. Just after the 20-minute time point the HR decreased precipitously as well, terminating with the presyncopal event. This is a typical Bezold-Jarisch pattern of presyncope. Note in the right hand column that treatment of this subject with midodrine one hour prior to the tilt-stand test was not associated with a decrease in BP and HR.

Figure 9. Kaplan-Meier presyncope-free survival curves of subjects treated with midodrine 5 mg one hour prior to the tilt-stand test and untreated control subjects (p = 0.0363).



Kaplan-Meier analysis of presyncope-free survival data is shown in Figure 9. Following head-down tilt bed rest, subjects who were treated with midodrine one hour prior to the tilt-stand test had a 71.4% rate of presyncope-free survival, whereas untreated control subjects had only a 25% rate of syncope free survival (p = 0.036).

Figure 10. Group average percent increase in calf size following successive application 30, 40, and 50 mmHg pressure around the thigh using an inflatable thigh cuff and rapid inflation device (Hokanson, Inc.)



In addition to increasing peripheral arterial resistance, midodrine is known to have a strong venous constricting effect as well. Shown in Figure 10 is the increase in calf size in response to a graded increase in circumferential pressure applied to the thigh. These data are consistent with an increase in venous compliance and transudation of fluid into the interstitial space

following bed rest, and provide further evidence that support the beneficial role of midodrine as a post-flight countermeasure against orthostatic intolerance.

IMPLICATIONS FOR FUTURE RESEARCH

Space Flight

The development of orthostatic intolerance is one of the primary cardiovascular risks and is a current operational problem for NASA. This study has contributed to the detailed understanding of mechanisms that lead to the development of post-flight orthostatic intolerance. In addition, it has identified a pharmacologic countermeasure which appears to be highly effective in reducing orthostatic intolerance. This research project dealt with male subjects who were primarily young adults. In future research we will study premenopausal women who are more susceptible to orthostatic intolerance as well as to study the effects of age on orthostatic intolerance. In addition, we propose to perform CSI measurements on astronauts pre and post flight as well as to test midodrine in astronauts as a countermeasure to the development of post-flight orthostatic hypotension.

We now believe that many of the cardiovascular alterations associated with space flight may be appropriate. The problem is the readaptation to a gravitational environement. The problem with many past proposed countermeasures, such as the intermittent application of LBNP during flight, are that they interfere with the appropriate adaptation of the cardiovascular system to the microgravity environment. The advantage of the midodrine countermeasure is that it is only applied immediate prior to reentry or ;r landing, and thus does not affect the cardiovascular system during space flight itself.

Earth Benefits

The CSI methodology used in this project may be applied to patients with diseases that affect closed loop cardiovascular regulation such as heart failure, diabetes and hypertension. This CSI methodology can be used both to study disease processes as well as to monitor patients and guide treatment. These studies also have implications for the use of midodrine to treat patients with orthostatic intolerance on earth.

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APPENDIX A – Project Research Data

Contained in this report and in publications listed in Appendix B

APPENDIX B – Publications

Mullen TJ, Berger Rd, Oman CM and Cohen RJ. Human Heart Rate Variability Relation is Unchanged During Motion Sickness. Journal of Vestibular Research 1998;8(1):95-105.

Mukkamala R, Mathias JM, Mullen TJ, Cohen RJ and Freeman R. System Identification of Closed-Loop Cardiovascular Control Mechanisms: Diabetic Autonomic Neuropathy. Am. J. Physiol. 1999;276 (Regulatory Integrative Comp Physiol. 45):R905-912.

Mullen TJ, Ramsdell CD, Sundby GH, Williams GH, Cohen RJ. Cardiovascular System Identification of Alterations in Cardiovascular Regulation During Simulated Space Flight. Proceedings. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999, League City, Texas pp. 290-292.

Chen, Chun-Cheng. Automated Cardiovascular System Identification. Master of Engineering in Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 2000.

Maa, Ming-Hokng. Alterations in Cardiovascular Regulation and Function Assessed Using Cardiovascular System Identification. Master of Engineering in Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 2000

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

1. Project Title: *Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity*

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EXECUTIVE SUMMARY

The volume regulating systems are integrated to produce an appropriate response to both acute and chronic volume changes. Their responses include changing the levels of the hormones and neural inputs of the involved systems and/or changing the responsiveness of their target tissues. Weightlessness during space travel produces a volume challenge that is unfamiliar to the organism. Thus, it is likely that these volume regulatory mechanisms may respond inappropriately, *e.g.*, a decrease in total body volume in space and abnormal responses to upright posture and stress on return to Earth. A similar "inappropriateness" also can occur in disease states, *e.g.*, congestive heart failure. While it is clear that weightlessness produces profound changes in sodium and volume homeostasis, the mechanisms responsible for these changes are incompletely understood. Confounding this analysis is sleep deprivation, common in space travel, which can also modify volume homeostatic mechanisms.

The purpose of this project is to provide the required understanding and then to design appropriate countermeasures to reduce or eliminate the adverse effects of microgravity. To accomplish this we are addressing five Specific Aims: 1) To test the hypothesis that microgravity modifies the acute responsiveness of the renin-angiotensin-aldosterone system (RAAS) and renal blood flow; 2) Does simulated microgravity change the circadian rhythm of the volume-regulating hormones?; 3) Does simulated microgravity change the target tissue responsiveness to angiotensin II (AngII)?; 4) Does chronic sleep deprivation modify the circadian rhythm of the RAAS and change the acute responsiveness of this system to posture beyond what a microgravity environment alone does?
Because the renin-angiotensin system (RAS) plays a pivotal role in blood pressure control and volume homeostasis, it likely is a major mediator of the adaptive cardio-renal responses observed during space missions and will be a special focus of this project. Thus, the overall goal of this project is to assess the impact of microgravity and sleep deprivation in humans on volume-regulating systems. To achieve this overall objective, we are evaluating renal blood flow and the status and responsiveness of the volume-regulating systems (RAAS, atrial natriuretic peptide and vasopressin), and the adrenergic system (plasma and urine catecholamines) in both simulated microgravity and normal gravity with and without sleep deprivation. Furthermore, the responses of the volume homeostatic mechanisms to acute stimulation by upright tilt testing, standing and exercise are being evaluated before and after achieving equilibrium with these interventions.

This work has implications for the treatment and prevention of maladaptive hemodynamic responses experienced by astronauts in flight and on return to Earth. It will increase our understanding of the mechanisms by which weightlessness and sleep deprivation change plasma volume and sodium homeostasis, thereby, providing entree to develop appropriate countermeasures.

Renal and Cardio-Endocrine Responses in Humans to Simulated Microgravity

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I. Project Research Activity

Hypotheses

There are two overarching hypotheses:

- Simulated microgravity disrupts the renal and cardio-endocrine systems responses to steady state and acute stress with these disruptions being more profound in the salt loaded than the salt depleted state.
- Sleep deprivation will have an additive adverse effect to microgravity on the responsiveness and regulation of the renal and cardio-endocrine homeostatic systems.

Specific Aims

The volume-regulating systems are integrated to produce an appropriate response to both acute and chronic volume changes. Their responses include changing the levels of the hormones and neural inputs of the involved systems and/or changing the responsiveness of their target tissues. Weightlessness during space travel produces a volume challenge, which is unfamiliar to the organism. Thus, it is likely that these volume regulatory mechanisms may respond inappropriately, *e.g.*, a decrease in total body volume in space and abnormal responses to upright posture and stress on return to Earth. A similar "inappropriateness" also can occur in disease states, *e.g.*, congestive heart failure. While it is clear that weightlessness produces profound changes in sodium and volume homeostasis, the mechanisms responsible for these changes are incompletely understood. In part, our ignorance is secondary to problems in studying these issues in space. Therefore, land-based studies have attempted to unravel these mechanisms by using models, which presumably simulate weightlessness, *i.e.*, chronic bed-rest. However, substantial deficiencies exist in the available data. For example, 1) studies usually have focused on cardiovascular rather than renal and hormonal responses; 2) few studies have examined the effect of acute stress superimposed on weightlessness; 3) no studies have considered the potential role of sleep deprivation as an additional contributing factor; 4) the role weightlessness *per se*

has on the circadian rhythms of the hormones involved has only occasionally been assessed; and 5) despite the fact that these are derangements in volume homeostatic systems, the effect of a sodium intake has not been explored.

Thus, the overall goal of this proposal is to assess the impact of simulated microgravity and sleep deprivation in humans on volume-regulating systems. To achieve this overall objective, we will evaluate renal blood flow and the status and responsiveness of the volume-regulating systems (the RAAS, atrial natriuretic peptide (ANP) and vasopressin) and the adrenergic system (plasma and urine catecholamines) in both simulated microgravity and normal gravity with and without sleep deprivation. Our Specific Aims are:

1: To test the hypothesis that microgravity modifies the acute responsiveness of the RAAS and renal blood flow. The hormonal response to changes in posture and renal blood flow in a tightly controlled environment will be the primary endpoints assessed. These parameters will be evaluated before and after ten days of bed-rest and before and after three acute stimuli – upright posture and exercise.

2: Does simulated microgravity change the circadian rhythm of the volume-regulating hormones? Some data suggest that it might. However, data from studies where the dietary and posture environment were tightly controlled are not available.

3: Does a simulated microgravity change the target tissue responsiveness to AngII? Modifying dietary sodium intake changes the response of target tissues to AngII. The status of the RAAS in microgravity mimics the low salt state, yet the subjects are on a moderate salt diet. Whether the target tissue responses are changed is unknown.

4: Does chronic sleep deprivation modify the circadian rhythm of the RAAS and change the acute responsiveness of this system to posture beyond what a microgravity environment alone does? As in Specific Aim 1, acute responsiveness of the RAA axis will be the primary end point.

We have conducted two pilot study protocols relevant to the current study. Five healthy male volunteers participated in each study. In each protocol, a supine-to-stand test was conducted in which plasma renin and aldosterone were measured intermittently during 160 minutes after subjects stood from a supine posture. In the first protocol, supine-to-stand tests were conducted before and after five days of continuous horizontal bedrest (a surrogate for weightlessness). In this protocol, the diurnal variation of plasma renin activity was also monitored before and after bedrest. In the second protocol, supine-to-stand tests were conducted before and after a five-day period of combined horizontal bedrest and sleep deprivation during which subjects were restricted to 6.1 hours of sleep per night.

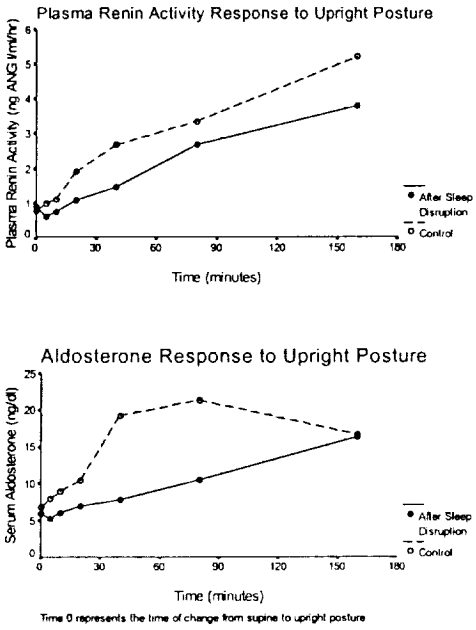


Figure 1: Effect of Sleep Disruption

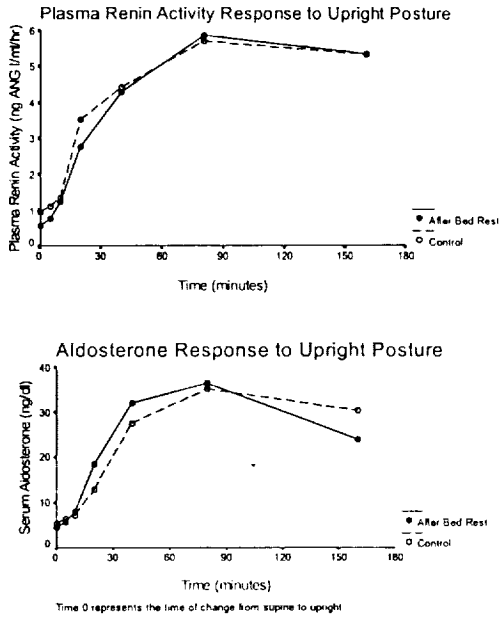


Figure 2: Effect of Bedrest

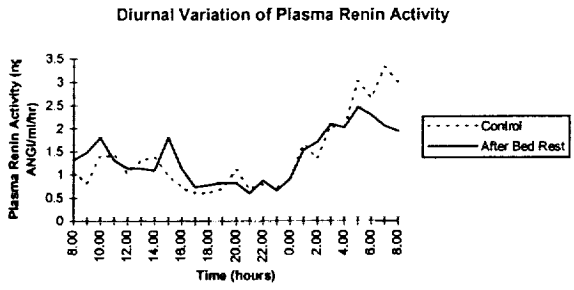


Figure 3: Diurnal variation of renin. Effect of Bedrest.

Statistical analyses of the results of these pilot studies, shown graphically in figures 1-3, suggest that prolonged bed-rest and particularly sleep disruption substantially alter the normal relationship between the renin-angiotensin system and aldosterone secretion. Renin-angiotensin levels in response to upright posture remain unchanged after either of these perturbations. However, aldosterone response to the increase in renin was significantly delayed in the sleep-deprived subjects who remained in the supine position for five days. In contrast, the circadian rhythm of renin was not disrupted. Thus, these results provide entree to a better understanding of the potential role of changes in volume homeostatic mechanisms in space flight that could account in part for the

cardiovascular instability upon return to one G. Either or both sleep deprivation and microgravity may contribute to this. The current study will provide data to help clarify the relationship of these factors with direct measurements of cardiovascular stability and reactivity.

Overall Design

Each of the two experimental protocols consists of four phases. Phase 1 involves implementing a controlled diet for three -five days in an inpatient setting. Phase 2 begins a set of basal measurements and measurements during the application of acute stressors made in a rigidly controlled environment. In Phase 3, the chronic environmental change, simulating the environment of space travel, is imposed. Finally, in Phase 4, the set of basal and acute stressor studies is repeated.

Facilities

General Clinical Research Center (GCRC) – Brigham and Women's Hospital. The GCRC of the Brigham and Women's Hospital was established in 1961. It provides extensive support for inpatient and outpatient studies and statistical, laboratory, and dietary components of patient-oriented and applied research projects. The current five year budget, which was activated December 1, 1994, is $22 million. The inpatient component is located on the ninth floor patient tower of the Brigham and Women's Hospital. All beds in the newly renovated GCRC (five IPM and eight other GCRC beds) are wired for continuous EEG, ECG, and temperature recordings using either Alice or Ultrasom systems. Its Core Laboratory (~1,800 sq. ft.) performs a wide range of hormonal assays. The Ambulatory General Clinical Research Center (~4,000 sq. Ft.) has five beds and utilizes a full-time research nurse. Other facilities on the GCRC include a clinical centrifuge, a cold room for special handling of blood following collection, and two metabolic kitchens for preparation of required measured diets. The Scientific Advisory Committee of the GCRC reviews proposals and makes recommendations to investigators and trainees on study design. The highly skilled nursing staff is associated with both the inpatient and outpatient components of the GCRC and performs a wide spectrum of techniques in clinical investigation.

Ambulatory General Clinical Center. The Ambulatory GCRC, located in more than 3,000 sq. ft. of space, consists of a waiting room, ten examination rooms, a nurses' office, two infusion rooms, a procedure room, a metabolic kitchen, a patient dining area, a conference room, and a small laboratory/sample processing area. A research nurse and secretary during regular business hours staff the Ambulatory GCRC. This facility is used to assist our research staff in screening procedures, which include physical examinations, electrocardiograms (ECGs), and the administration of additional tests required in the selection of subjects for the inpatient protocol. It is also available for any post-hospitalization studies that may be required.

Core Laboratory. The Core Laboratory of the GCRC was established in 1961 and currently is the largest Core Laboratory supported by the National Institutes of Health

GCRC program. Currently 40 analytes are measured in the Core Laboratory. GCRC investigators process more than 50,000 assays per year. An individual investigator cannot use more than 5% of the GCRC's Core Laboratory's budget. However, users with a large number of assays still can draw on the expertise and cost savings of this facility by paying the prorated cost of those samples above this limit. In most circumstances, this is a contribution of personnel and supply costs from primary project budgets. In it's extended capacity (with additional users of the GCRC and a physical merger with a Core Laboratory for a Specialized Center of Research in Hypertension (SCOR) program supported by NIH), more than 100,000 assays are processed per year.

Computerized Data-Based Management and Analysis Systems (CDMAS). The GCRC has one of the longest involvements with a CDMAS resource. The GCRC also has been the leader in developing innovative uses for computer handling of large data bases. CDMAS personnel are responsible for the maintenance of the many pieces of electronic monitoring equipment in the IPM facility, data storage, and data transfer to investigators for appropriate analysis. Additionally, the GCRC provides support for a biostatistician.

Core Research Protocols

Rationale: While there are no perfect ground-based models of weightlessness in a controlled environment where a variety of physiologic factors can be assessed simultaneously, the one that has been most commonly used is prolonged head down tilt bed-rest. Protocol 2, which is identical to Protocol 1, except that subjects sleep only 6 hours per night instead of 8 hours, will be initiated in the final quarter of Year 2.

Experimental Subjects: Healthy male volunteers are recruited for study. Only subjects who have provided written, informed consent for their participation are considered. Those volunteers who meet all of the screening criteria are selected. Sample size estimates were derived from what is assumed to be the least sensitive and/or precise of the experimental techniques. Estimates were calculated assuming type I and II error probabilities of 0.05. Since both measurements during control and simulated space flight conditions are made for each subject, paired analysis is appropriate. From previous data, the procedure that was assumed to be the least sensitive to the microgravity was the plasma renin activity response to upright posture. Given the variability of the response characteristic in normal subjects on a high sodium diet and assuming that only a 20% change in the time response curve would be of physiologic significance, data from 12 patients would be required. Based on our preliminary experience with these types of studies, we anticipate about a 15% loss rate for individual data either because of incomplete studies, lost samples, or laboratory errors. Thus, 14 subjects are needed for this protocol.

Experimental Procedure: After completion of the screening procedures, subjects will begin the first of four phases of their study. Phase 1 lasts three-five days and takes place in the GCRC where they begin a constant, isocaloric, diet consisting of 200 mEq sodium, 100 mEq potassium, and 2500 mls fluid. This phase equilibrates the subjects in terms of caloric and electrolyte intake, since these factors can modify hormonal, renal, and cardiac responses. Throughout their in-patient course, the subjects are maintained

on a constant light (16 hour)/dark (8 hour) cycle. (Light/dark cycle is 18/6 hours for subjects under sleep deprivation protocol). They are not permitted to doze during their light cycle, which begins at 6:00 am. In addition, routine vital signs and daily weights are recorded every 8 hours. During Phase 2, the subjects continue the diet and 24 hour urine samples are collected daily for the duration of the hospital stay. Total volume, creatinine, sodium, potassium, calcium, magnesium, chloride, phosphate, bicarbonate, and cortisol are measured daily with additional measurements at selected times (see Experimental Measurements). During Phase 2 of the protocol in the GCRC environment all of the measurements listed below in the section Experimental Measurements, as well as those listed in the section Acute Stressors, are made to establish pre-head down tilt bed-rest baseline values.

On completion of Phase 2, the subjects begin Phase 3, which is the maintenance of head down tilt bed-rest for sixteen days. During the final two days of head down tilt bed-rest all of the measurements listed below in the section Experimental Measurements are repeated.

Phase 4 is the recovery phase, which lasts three days. During this phase, the subjects are allowed ad lib activity but must continue the constant diet. On the morning of the first and third days of Phase 4, the acute interventions are repeated. Thus, the impact of simulated microgravity on basal levels of the cardiac, vascular, renal, and endocrine systems are assessed as well as their responses to the acute interventions. Two control periods, one before and one after the simulated microgravity phase, are used.

Experimental Measurements

1) *Urine Collections.* Twenty four hour urine assessment of creatinine, sodium, potassium, calcium, magnesium, chloride, phosphate, and cortisol are obtained on each day of the study. On the last days of the control and perturbation phases and on the first and second days of the recovery phases, catecholamines are also measured.

2) *Renal Blood Flow.* Measuring para-aminohippurate (PAH) clearance assesses renal blood flow. A PAH loading dose (12 mg/kg) is administered acutely, and then a continuous infusion of PAH is maintained for the duration of the study. After a 60 minute equilibration period, three blood samples are obtained five minutes apart for PAH level determination. PAH levels are measured using an automated system. Glomerular filtration rates (GFRs) are measured by the creatinine clearance technique.

3) *Supine to Upright Posture Study.* Subjects are woken up at 06:00 and remain supine until 10:00. At 120, 10, and 2 minutes prior to the beginning of upright tilt, blood samples are obtained. At 10:00, the subject is tilted upright on a tilt table to thirty degrees for ten minutes. The angle of tilt is then increased to sixty degrees for ten minutes. Finally the subject stands upright quietly with additional blood

samples obtained from an indwelling catheter at repeated intervals until 12:40. Plasma renin activity, aldosterone, cortisol, norepinephrine, epinephrine, and dopamine are measured from these blood samples. This technique allows us to assess the acute responsiveness of this axis to the stress of upright posture.

4) *Upright Bicycle Exercise.* Exercise is performed on a computer controlled bicycle ergometer. The exercise protocol involves graded increases in pedaling resistance until a heart rate of 70% of maximum predicted heart rate is achieved. Heart rate is sustained at or above a minimum rate of at least 105 beats per minute for at least 256 beats. Blood samples are drawn prior to exercise, and at the end of the exercise for plasma renin activity, aldosterone, cortisol and catecholamines. During the period of exercise surface electrodes (CH 2000 multi-segmented electrode set) are used to record ECG signals for determination of microvolt level T wave alternans analysis as described in Annual Reports of Professor Cohen.

5) *Plasma Renin Activity, Aldosterone, Cortisol, Atrial Natriuretic Peptide, Vasopressin, Catecholamines, sodium, potassium, calcium, magnesium, chloride, and phosphate.* These hormones are measured by standard techniques within the Core Laboratory on the last days of the control and perturbation phases and the first and second days of the recovery phases.

6) *Angiotensin Infusion Test.* Angiotensin II amide (Ciba, Summit, N.J.) is infused at rates of three and six ng/kg/min for 30 minutes after a 60 minute control period. A non-invasive Dinamap device records blood pressure and pulse every two minutes. Aldosterone levels are obtained at the end of the control period and each dose periods.

7) *Norepinephrine Infusion Test.* Norepinephrine is infused at rates of 30, and 60 ng/kg/min for fifteen minutes at each dose. Blood pressure and pulse are recorded every two minutes using a non-invasive Dinamap device.

Results

Specific Aim 1: Microgravity influences volume status and volume regulating hormones.

A clearly augmented PRA and aldosterone response to the assumption of upright posture following bed-rest was documented, which is also consistent with an increased tone of the renin-angiotensin-aldosterone hormonal axis in response to simulated weightlessness **(Figure 1)**. The basal levels of both PRA and aldosterone were also increased on the final day of bed rest when compared with pre-bed rest, [PRA, (50% increase) relatively more than aldosterone (10% increase)] but reverted to levels at or below pre-bed rest levels on the first and second ambulatory days following the bed rest period **(Figure 2).**

Strikingly, there was a pronounced age-dependent effect on these parameters (**Figure 3**). Subjects less than 30 years old had significantly reduced basal levels and responsiveness.

Figure 1. Group average Plasma Renin Activity (PRA) and Aldosterone responses to upright posture pre- bed rest (day 5) versus post-bed rest (day 21). Upright tilt began at a relative time of 0. "Postex" indicates the values after finishing a bicycle exercise protocol that was part of a parallel study of susceptibility to ventricular arrhythmias (Mean ± SEM).

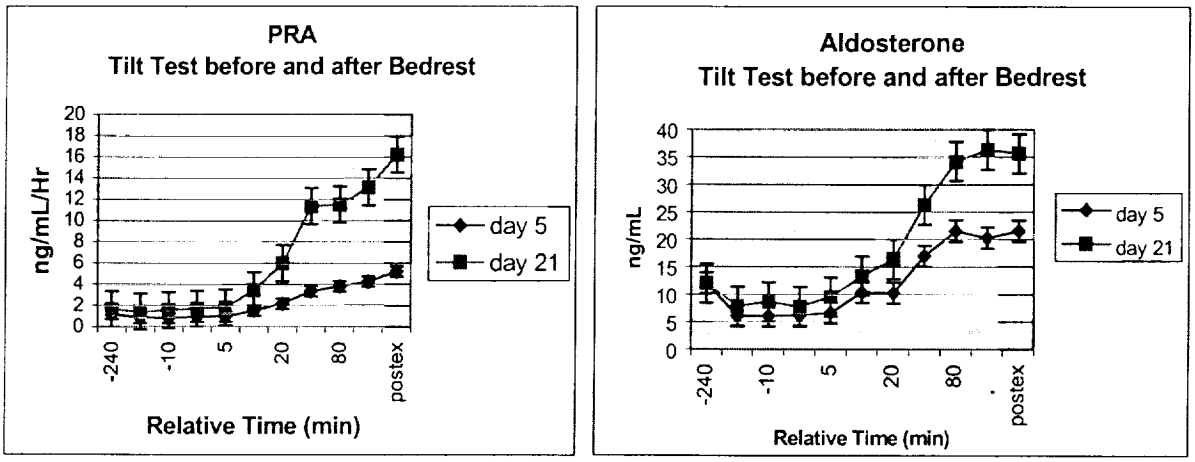


Figure 2. Group average PRA and aldosterone from blood drawn at 6:00 a.m. pre-bed rest (day 5) versus at 6:00 a.m. on the final day of bed rest before being tilted upright (day 21), and on the first and second ambulatory days (days 22 and 23) (Mean ± SEM).

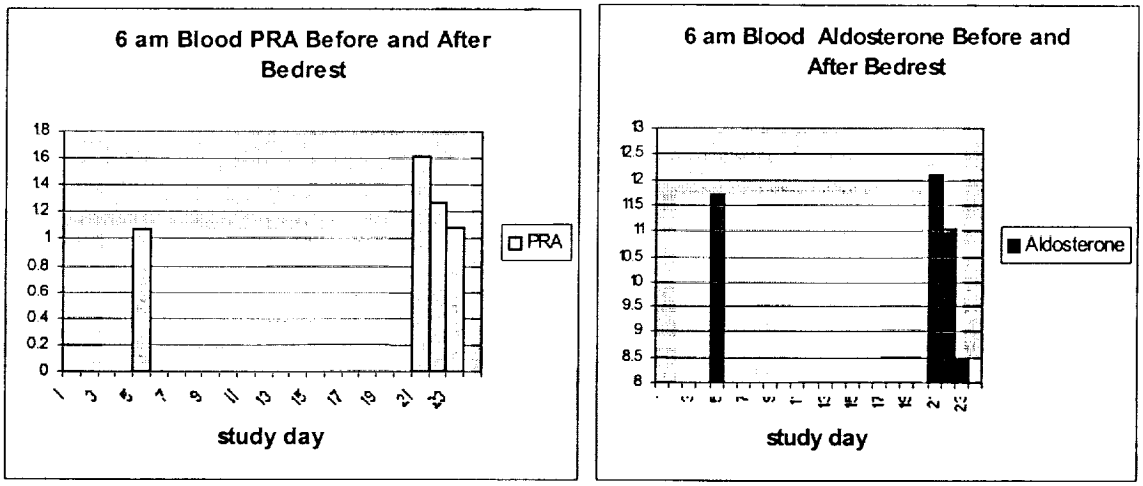
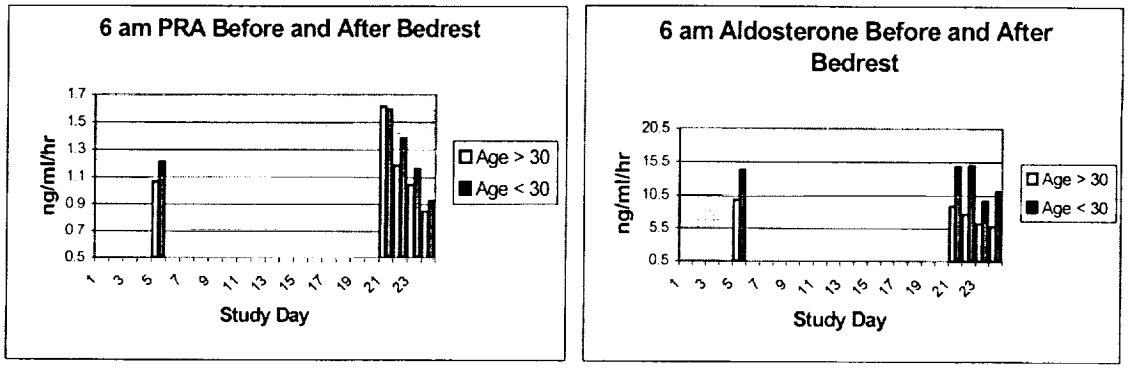
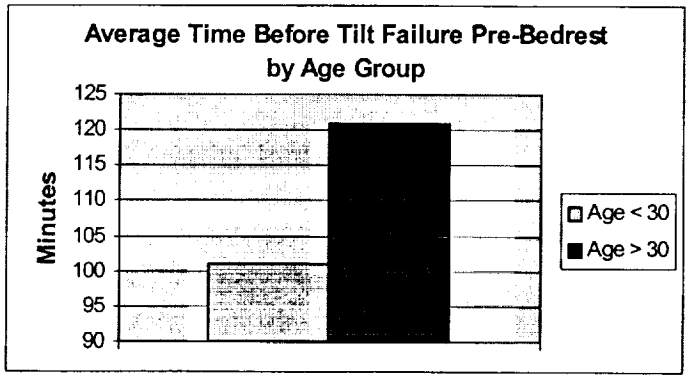


Figure 3. Average PRA and aldosterone from blood drawn at 6:00 a.m. pre-bed rest (day 5) and. on the final days of bed rest before being tilted upright (days 21) and on the ambulatory days (days 22, 23 and 24) in older (>30 y.o.) and younger (<30 y.o.) groups.



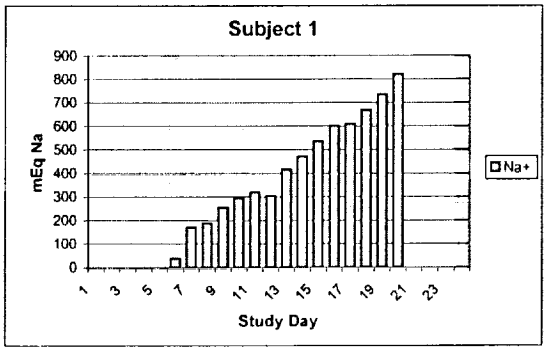
Interestingly, a different susceptibility to orthostatic intolerance prior to bed-rest appears to correlate with these differences in the responsiveness of the RAAS to upright posture, with the younger subjects having poorer pre-bed rest orthostatic tolerance, as can be seen in **(Figure 4)**. Both the differences in RAAS activity and orthostatic intolerance appeared to be further exaggerated in subjects older than 40 years. However, the number of subjects was insufficient to be sure. Since these data were collected in conjunction with a randomized placebo controlled trial of midodrine as a countermeasure against post bed orthostatic hypotension, there are insufficient numbers of untreated control subjects to draw meaningful conclusions about differential susceptibility to post-bed rest orthostatic hypotension among these groups.

Figure 4. Comparison of tilt test tolerance in subjects younger than 30 versus those older than 30.

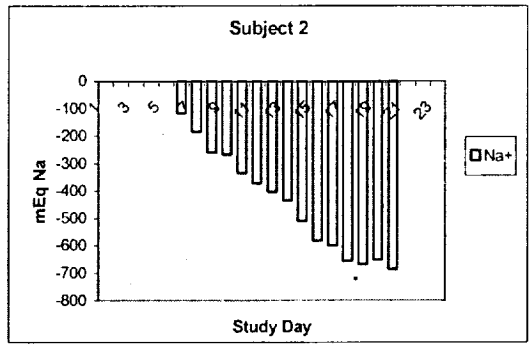


As was true of the activity of the RAAS, there was also substantial variability in how the subjects' sodium homeostasis responded to simulated microgravity. Some subjects avidly held on to sodium during simulated microgravity (**Figure 5a).** Others actively rejected it **(Figure 5b).**

Figure 5a and b. Cumulative sodium balance in two subjects undergoing the simulated microgravity protocol. Values were obtained by summing the daily differences between sodium intake and sodium excretion. Note that steady state balance was achieved in subject 2 by day 12 but never achieved by Subject 1. The initial and final bars denote the beginning and end of head down bed rest.



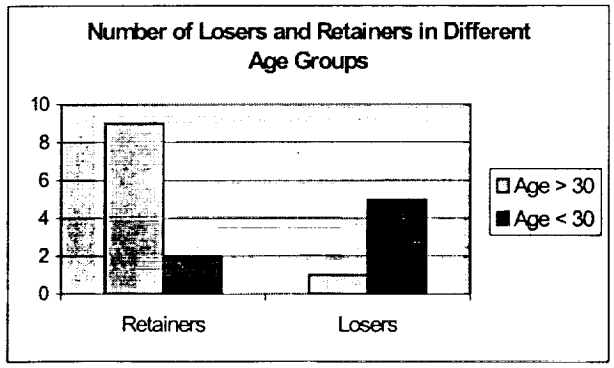
a



b

In most cases, subjects usually came into a new steady state within ten days of starting the bed rest protocol, particularly those who lost sodium. Age effected the likelihood of becoming either a retainer or loser. Using a cumulative sodium balance of $\pm$ 100 mEq as the "no change" rate, sixteen subjects could be characterized. Nearly 90% of subjects > 30 years of age were retainers, while only 30% of those < 30 years of age were losers which nearly reached statistical significance (p = 0.08). **(Figure 6).**

Figure 6. Number of subjects with positive cumulative sodium balance (retainers) and subjects with negative cumulative sodium balance (losers) by age group.



As might be anticipated, if subjects were divided into "retainers" or "losers", retainers were more tolerant to pre-bedrest, upright posture than losers (Figure 7), although this again did not reach statistical significance (p=0.11). Again, these differences were exaggerated in the younger subjects compared to those >40 years of age. Finally, there also was a suggestion of a difference in systolic, but not diastolic, blood pressure response to simulated microgravity with age (Figure 8).

Figure 7. Comparison of Pre-bed rest tilt test tolerance among retainers of sodium versus losers of sodium.

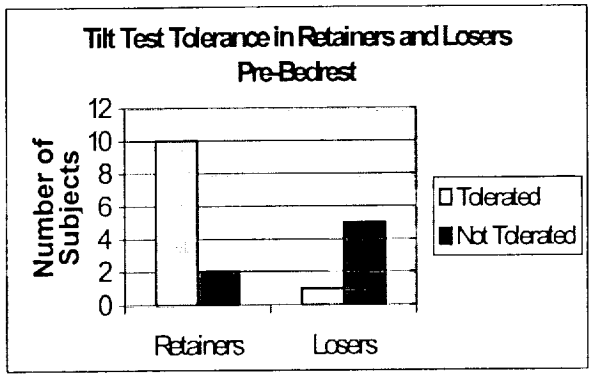
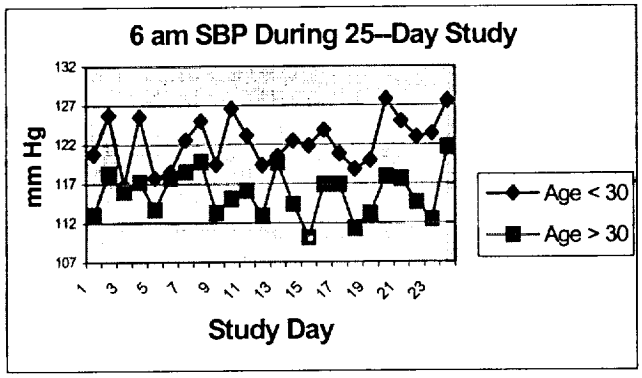


Figure 8. 6 am Systolic Blood Pressure in older and younger subjects.



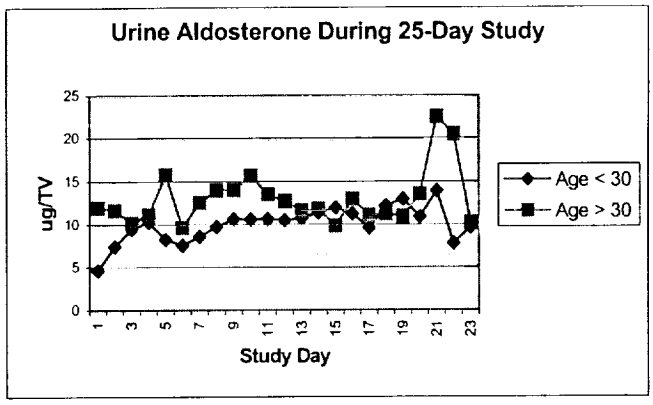
In the preliminary studies we also measured 24 hours aldosterone excretion rates, a better index of the activity of this system over time than serum levels **(Figure 9).**







Figure 9. Average 24-hours urine aldosterone in older (>30 y.o.) and younger (<30 y.o.) subjects. Tilt tests were performed on day 5 and day 21



In older subjects, excretion was significantly higher ($p = 0.015$) on the day of the first tilt study and remained elevated during the first seven days of simulated microgravity. Most striking was the aldosterone response to the second tilt study after bed rest, where older subjects had an even more profound elevation which, in contrast to the younger subjects persisted for 48 hours after resuming normal ambulation.

Thus, study results demonstrate that plasma renin activity (PRA) and aldosterone levels were augmented in response to upright posture following bed rest. This effect was found to be more pronounced in older subjects. Importantly, these differential responses appear to correlate with differential orthostatic tolerance, i.e. the older subjects with an augmented RAAS response to upright posture appear to have a better ability to tolerate orthostatic stress. We found that there were differential sodium handling characteristics in response to microgravity whereby those subjects who retained sodium aggressively during bed rest had a greater tolerance of orthostatic stress. In addition, there was a suggestion from these studies that this response correlated with age, i.e., older subjects tended to retain sodium more aggressively in response to bed rest and seemed to have greater orthostatic tolerance following exposure to microgravity. This difference may be a function of differential renal sodium handling, or of age, or of both.

Specific Aim 2. Circadian Rhythm and Microgravity.

Twenty-four hour circadian rhythms of pulse, aldosterone, cortisol and plasma renin activity were determined in ten subjects studied prior to and following fourteen days of head-down tilt. No shift in rhythms was observed.

Specific Aim 3. Target tissue responses to vasoconstrictors.

Nineteen subjects received infusions of Angiotensin II and Norepinephrine before and after simulated microgravity. The following factors were assessed: aldosterone, cortisol and pulse and blood pressure response to Angiotensin II and blood pressure and pulse response to Norepinephrine. Microgravity did not influence any of these except the aldosterone response to Angiotensin II. This response was similar to that shown in Figure 2 for tilt responses.

Specific Aim 4. Sleep Deprivation.

To date eight subjects have enrolled in this phase. Data from only two subjects are available. Therefore, no conclusions can be drown yet.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

As was mentioned above, study results demonstrated that augmentation of PRA and aldosterone levels in response to upright posture following bed rest was found to be more pronounced in older subjects. In addition, older subjects tended to retain sodium more aggressively in response to bed rest. They also demonstrated to have greater orthostatic tolerance following exposure to microgravity. This difference may be a function of differential renal sodium handling, or of age, or of both. To clarify this issue we plan to study a population of men over 50 years of age using the same protocol used in our previous study. These data will be compared with the data set generated in predominantly younger men (<35 years of age) to test the hypothesis that age will modify the response pattern of the RAAS following microgravity exposure and that these modifications correlate with orthostatic tolerance. The hormonal response to changes in posture in a tightly controlled environment will be the primary endpoints assessed. These parameters will be evaluated before and after sixteen days of bed-rest and before and after two acute stimuli – upright posture and exercise.

We did not study woman subjects under the current protocol because of cyclic hormonal changes, which would require specific modifications in protocol design. It has been observed that orthostatic intolerance following spaceflight is more pronounced among women than among men. To clarify this issue, we plan to study a population of menstruating females using the same protocol used in our previous study to test the hypotheses that females have the same patterns of renal sodium handling in response to microgravity as do men, but have less orthostatic tolerance.

Appendixes:

A. See results section (p.9)

B. None

C. 3 papers submitted to JAP

NSBRI FINAL REPORT: Inclusive Dates 10/01/1997 to 9/15/2000

1. **Project Title:** Rodent Studies of Cardiovascular Deconditioning

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Changes in blood pressure can occur for two reasons: 1) A decrease in cardiac output resulting from the altered contractility of the heart or through changes in venous filling pressure via the Frank Starling mechanism or; 2) A change in systemic vascular resistance. The observed changes in cardiac output and blood pressure after long term long term space flight cannot be entirely explained through changes in contractility or heart rate alone. Therefore, alterations in filling pressure mediated through changes in systemic venous capacitance and arterial resistance function may be important determinants of cardiac output and blood pressure after long term space flight. Our laboratory has previously shown the importance of veno-constriction mediated by the carotid sinus baroreceptor reflex system on overall circulatory homeostasis and in the regulation of cardiac output.

Our proposed experiments test the overall hypothesis that alterations in venous capacitance function and arterial resistance by the carotid sinus baroreceptor reflex system are an important determinant of the cardiac output and blood pressure response seen in astronauts after returning to earth from long term exposure to microgravity. This hypothesis is important to our overall understanding of circulatory adjustments made during long term space flight. It also provides a framework for investigating countermeasures to reduce the incidence of orthostatic hypotension caused by an attenuation of cardiac output. We continue to use hind limb unweighted rat model to simulate the pathophysiological effects as they relate to cardiovascular deconditioning in microgravity. We have used this model to address the hypothesis that microgravity induced cardiovascular deconditioning results in impaired vascular responses and that these impaired vascular responses result from abnormal alpha-1 AR signaling. The impaired vascular reactivity results in attenuated blood pressure and cardiac output responses to an orthostatic challenge. We have used in vitro vascular reactivity assays to explore abnormalities in vascular responses in vessels from HLU animals and, cardiac output (CO), blood pressure (BP) and heart rate (HR) measurements to characterize changes in hemodynamics following HLU.

Specific Aims of Initial Proposal to NSBRI:

I. CONSCIOUS ANIMAL EXPERIMENTS:

A. Reflex Control of Stroke Volume and Cardiac Output.

We will test the hypothesis that the cardiac output and stroke volume response to carotid sinus stimulation in conscious normal (non-suspended) Sprague Dawley(SD) rats are different in magnitude than in conscious tail suspended rats. We will measure the arterial pressure and right atrial and venous pressures, heart rate, cardiac output(ascending aortic flow) and stroke volume in both normal and tail suspended rats.

II. ANESTHETIZED ANIMAL EXPERIMENTS:

A. Reflex Control of Total Systemic and Venous Capacitance

1. We will test the hypothesis that the changes in systemic venous capacitance caused by the carotid sinus baroreceptor reflex system are the same in the two types of rat. We will use our newly developed cardio-pulmonary bypass technique in the rat. This technique is similar the techniques we had previously used in the canine model to

measure changes in capacitance function; venous unstressed volume and venous compliance's.

2. We will test the hypothesis that the carotid sinus reflex control of the pressure-diameter relationships of micro-vascular venules in the two types of rats is the same. We will directly measure individual microvascular pressures and diameters in 20-200 micron vessels in the intestinal vascular bed. We choose the intestinal vascular bed because, we have previously shown that it is the primary location for vascular capacitance changes.

B. Mechanism of the Dysfunction of Control of Venous Capacitance

We hypothesize that there is an alteration in vascular responsiveness, which is associated with alpha-1 adrenoreceptors in the venous vascular bed of the intestine. We will use both in-vivo and in-vitro experimental models to test the functional changes in the venous alpha-1 receptors.

III. COUNTER MEASURES

We will begin to test several counter measures, both mechanical and pharmacological, to offset the reduction in cardiac output. With the consultation with NASA scientists (Drs. Susan Fortney and Janice Fritz-Yelle) and the rest of the animal model team, we will develop strategies to offset the loss of sympathetic venous tone.

Modifications Required because of Funding Limitations:

In recognition that the funding level of the proposal was drastically reduced from the requested $ 250,000 to $ 175,000 (total cost), we were forced to reduce the originally proposed scope. We had also directed our research efforts based on preliminary data obtained from experiments performed which will ultimately be the most important driving force to develop counter measures. One our primary hypotheses is that there is an alteration in the alpha-1 adrenoreceptor in the venous capacitance vessels in hind limb unweighted animals. We are utilizing both in vivo bilateral carotid artery occlusion and video microscopy of gut microvessels and as well as in vitro pressure dimension analysis to determine the functional response of microvessels to endogenous NE (in-vivo) and exogenous (NE and phenylephrine) alpha-1 AR stimulation. Much of the data that has been collected is given in detail within the submitted papers (see appendix)

Summary of Accomplishments:

Our proposed experiments test the overall hypothesis that alterations in venous capacitance function and arterial resistance by the carotid sinus baroreceptor reflex system are an important determinant of the cardiac output and blood pressure response seen in astronauts after returning to earth from long term exposure to micro-gravity. This hypothesis is important to our overall understanding of circulatory adjustments made during long term space flight. It also provides a framework for investigating counter measures to reduce the incidence of orthostatic hypotension caused by an attenuation of cardiac output. We continue to use hind limb unweighted (HLU) rat model to simulate the pathophysiological effects as they relate to cardiovascular deconditioning in micro-gravity. We have used this model to address the hypothesis that micro-gravity induced cardiovascular deconditioning results in impaired vascular responses and that these impaired vascular responses result from abnormal alpha-1 AR signaling. The impaired

vascular reactivity results in attenuated blood pressure and cardiac output responses to an orthostatic challenge.

We have used in vitro vascular reactivity assays to explore abnormalities in vascular responses in vessels from HLU animals and, cardiac output (CO), blood pressure (BP) and heart rate (HR) measurements to characterize changes in hemodynamics following HLU. Overall, we have been able to show that micro-gravity exposure is associated with a decrease in sympathetic neurotransmission (SN). This in turn is associated with a decrease in alpha-1 AR number and signaling as well as vessel smooth muscle mass (trophic effects of NE). Upon return to gravity, attenuated vascular contractility occurs secondary to end organ hyporesponsivenss, despite normal or accentuated sympathetic neurotransmission. Impaired venular and arteriolar responses to catecholamine stimulation results in impaired stroke volume, cardiac output and blood pressure responses. Specifically,

1. We have demonstrated impaired CO responses to an orthostatic challenge in rats following HLU which recovers in ~60hrs (attached manuscript: submitted, Am J Physiol)
2. We have demonstrated that after HLU, unstressed venous vascular volume is increased following HLU and can no longer decrease in response to sympathetic stimulation. This supports our primary hypothesis and may underlie the mechanisms leading to an exaggerated fall in stroke volume seen in astronauts (attached manuscript in press, J Appl Physiol)
3. Using cardiopulmonary bypass studies in which cardiac output is fixed, we have demonstrated that venous an total circulatory capacitance is increased following HLU (attached manuscript in press, J Appl. Physiol)
4. We have demonstrated impaired alpha-1-AR and non–alpha mediated responses in large arteries (aorta) of HLU animals. We have also demonstrated that the observed vascular contractile hyporesponsiveness is reversible with time, In addition, alpha-1AR specific abnormalities in mesenteric microvessel responsiveness appear to be present. (attached manuscript in review, J Appl. Physiol)
5. We have observed a decrease in alpha-1AR specific radioligand binding in aortic vessels from HLU animals.
6. We have demonstrated both an endothelial dependent and endothelial independent component which contributes to vascular hyporesponsiveness. following HLU.
7. We have demonstrated vascular hyporesponsiveness in the large pulmonary arteries of the HLU rats. This vascular hyporesponsiveness is obliterated with nitric oxide synthase inhibition suggesting that increased nitric oxide production may be mediating this impaired contractile response.
8. We have demonstrated an impaired heart rat and blood pressure response to a orthostatic stimulus (transient bilateral carotid occlusion) in a HLU mouse model.
9. We have developed an external non-invasive mechanical prototype device, in conjunction with the Applied Physics Laboratory of JHU, that peristaltically pumps blood from lower extremities and abdomen towards the heart to maintain stroke volume and cardiac output during an orthostatic challenges. A notice of invention and non-disclosure has been filed with Johns Hopkins University.

In general our data support the hypothesis that vascular and specifically venular hyporesponsiveness is likely to contribute to impaired stroke volume response and blood

pressure regulation following microgravity. We have also continued to validate the HLU rat model as a model that recapitulates the cardiovascular changes that occur following microgravity in humans. These accomplishments have allowed us to refine mechanisms, begin to test countermeasures, and bridge the gap between animal models and human subjects in our understanding of micro gravity induced orthostatic intolerance.

The future direction of our research is laid out in our competitive renewal and includes preliminary data we have collected addressing:

1) Cardiac mechanisms of cardiovascular deconditioning. We have begun to measure integrated cardiovascular function by recording pressure volume loops using micomanometric and conductance catheters in HLU rats. We are using magnetic resonance imaging of the heart to measure cardiac mass and determine whether HLU is associated with cardiac atrophy.

2) In order to address molecular mechanisms of vascular contractile hyporesponsiveness, we are investigating both endothelial dependent and independent mechanisms. We are measuring simultaneous force (vessel strips) and vessel diameter together with intracellular Ca2+ using fluorescence spectrophotometry in Fura-2AM (Ca2+ sensitive dye) loaded vessels. This will allow us to determine if the mechanism of vascular contractile hyporesponsivensss is due to abnormalities in Ca2+ regulation or the sensitivity of the contractile apparatus to Ca2+, or both.

3) Based on our previous work, one of the primary mechanisms of orthostatic intolerance involves impaired contractile responses in vascular tissue. Thus, countermeasures aimed at increasing the contractile response in the vasculature represents a novel approach to preventing orthostatic intolerance. Vascular contractile responsiveness may be enhanced by altering signaling pathways, at several different levels. We plan to test three specific sites which offer the potential for reversing hyporesponsivenss. These include: 1) Enhancing the impaired contractile response by administering pharmacological doses of an orla alpha-1 adrenergic receptor (alpha-1AR) agonist, midodrine. 2) Enhancing expression of alpha-1AR receptors that are downregulated by micro-gravity (Insulin Growth Factor (IGF-1 through administration of growth hormone (GH)). IGF-1 has been shown to increase the expression of alpha-1AR receptors in vascular smooth muscle cell culture. We have demonstrated an attenuated vascular response to alpha-1AR agonists and a decrease in alpha-1AR number in our model. Thus we plan to test the effect of GH (which mediates its effects through IGF-1 on vascular function, and alpha-1AR expression. 3) Enhancing the sensitivity of the contractile apparatus to prevailing $[Ca^{2+}]i$ by inhibition of the enzymes that are critical in the desensitization of the contractile apparatus (antisense oligonucleotides to decrease expression of myosin light chain phosphatase (MLC-P) expression.

Publications:

Papers: All nine papers are enclosed

1. McKeown K., Shoukas A. Chronic isolation of the carotid sinus baroreceptor region in conscious normotensive and hypertensive rats. Am J Physiol 275 (Heart and Circ Physiol 44) H322-H329, 1998
2. Shapiro R., Winters B., Hales M., Barnett A., Schwinn DA., Flavahan N., Berkowitz D. Circulating sympatholytic factor in a patient with sympathotonic orthostatic hypotension. Hypertension (In Press)
3. Winters B, Mo, Z., Brooks-Asplund E, Shoukas A, Nyhan D, and DE Berkowitz. Leptin reverses endothelial dysfunction in leptin deficient ob/ob mice. Journal of Applied Physiology (In Press)
4. Dunbar, S.L., D.E. Berkowitz, E.B. Brooks-Asplund, and A.A. Shoukas. The effects of hindlimb unweighting on the capacitance of rat small mesenteric veins. Journal of Applied Physiology (In press)
5. Dunbar, S.L., L. Tamhidi, D.E. Berkowitz, and A.A. Shoukas. Hindlimb unweighting affects rat vascular capacitance function. American Journal of Physiology (In press)
6. Brooks-Asplund, E., Shoukas A. Baroreceptor contribution to the cardiovascular reflex responses of phenylephrine and sodium nitroprusside in the conscious rat. American Journal of Physiology (In Review)
7. Berkowitz D, Marucci L, Winters B, Asplund E, Nyhan D, Shoukas A. Impaired but reversible vascular reactivity in a rat model of micro-gravity. Journal of Applied Physiology (In Review)
8. Brooks-Asplund, E., Berkowitz DE, Dunbar, S. and A.Shoukas. Hindlimb unweighting attenuates cardiac output and stroke volume responses to upright tilt in male rodents. American Journal of Physiology (In Review)
9. Jung, A.S., Berkowitz, D., Brooks-Asplund, E.M., Dunbar, S. and A. Shoukas. Contribution of Carotid and Aortic Baroreceptors to Heart Rate and Pressure Changes in Acute Mice. American Journal of Physiology (Submitted)

Abstracts:

10. Berkowitz D., Marrucci L., Winters B., Nyhan D., Szumski A., Shoukas A. Vascular response in a rat model of micro-gravity. Circulation 98(17):658, 1999.
11. Brooks-Asplund, E., Shoukas A. Baroreceptor contribution to the cardiovascular reflex responses of phenylephrine and sodium nitroprusside in the conscious rat. FASEB 373.11, 1999.
12. Berkowitz DE, Winters B, Mo Z, Brooks-Asplund E, Shoukas A, Nyhan D. Abnormal vasodilator responses in leptin deficient ob/ob mice. Amer Heart Assn 72nd Scientific Session, Atlanta, GA, November 1999.
13. Berkowitz DE, Brooks-Asplund E, Mo Z, Winters B, Nyhan D, Shoukas A. Impaired mesenteric micro vascular reactivity and reversibility in rat model of micro-gravity. Amer Heart Assn 72nd Scientific Session, Atlanta, GA, November, 1999.
14. Kim SY, Berkowitz DE, Jhaveri R, Shoukas A. and D. Nyhan. Differential influence of pulmonary vasoconstrictors on milrinone induced vaso relaxation. Society for Cardiovascular Anesthesiology, 2000.

15. Brooks-Asplund, E., Berkowitz DE, Dunbar, S. and A.Shoukas. Cardiovascular Responses to Orthostatic Challenge Before and After Hind limb-Un-weighting (HLU) in Male Rats. FASEB 2000.
16. Jung, A.S., Berkowitz, D., Brooks-Asplund, E.M., Dunbar, S. and A. Shoukas. Contribution of Carotid and Aortic Baroreceptors to Heart Rate and Pressure Changes in Acute Mice. FASEB 2000.
17. Berkowitz, D. E., Kim, S.Y., Brooks-Asplund, E.M., Nyhan, D. and A. Shoukas. Myosin Light chain Phosphatase(MLC-P): A target for enhancing vascular contractile responses in cardiovascular deconditioning: 73 Scientific Session, American Heart Association, New Orleans, LA, 2000
18. Kim, S.Y., Berkowitz, D. E., Dunbar, S, Nyhan, D. and A. Shoukas. Impaired contractile responses in pulmonary artery in a rat model of microgravity: Role of nitric oxide 73 Scientific Session, American Heart Association, New Orleans, LA, 2000

The above abstracts were also presented at both NSBRI conferences in Texas.

National Space Biomedical Research Institute

FINAL PROJECT REPORT

PROJECT TITLE: *Computational Models of the Cardiovascular System and its Response to Microgravity*

Cardiovascular Alterations Team

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EXECUTIVE SUMMARY

Computational models of the cardiovascular system are powerful adjuncts to ground-based and in-flight experiments. They provide a rational framework that quantitatively defines interactions among complex cardiovascular parameters, supports the critical interpretation of experimental results and testing of hypotheses, and permits prediction of the impact of specific countermeasures.

Over the past 3 years we have implemented a computational model of the cardiovascular system capable of simulating the short term (0-5 min), transient response to orthostatic stress tests such as tilt/standing and lower body negative pressure (LBNP) in normals and microgravity adapted individuals. The model consists of a lumped parameter representation of the hemodynamic system and set-point representations of the cardiopulmonary and the arterial baroreflexes. The model allows for regional blood pooling in four systemic circulatory branches and three central venous compartments. Furthermore, we implemented non-linear venous pressure-volume characteristics for all dependent venous vascular compartments and allowed blood volume to change as a function of time and orthostatic stress to simulate blood plasma sequestration into the interstitium during orthostatic stress. We accounted for a reduction in the hydrostatic pressure component at the carotid sinus during tilt by making the input pressure to the arterial baroreflex a function of orientation in the gravitational field.

We verified the model under baseline (supine) conditions and after three minutes of orthostatic stress by comparing the model's predictions to a limited set of population-averaged data found in the medical literature. Figure 1 shows the hemodynamic responses following sudden tilts to varying angles of elevation. We also studied the transient response of the cardiovascular system to sudden gravitational stress. Figure 2 shows the heart rate response to standing in an astronaut before spaceflight. As can be seen from Figures 1 and 2, the experimental steady state and transient responses are well matched by the simulator.

By appropriately modifying some of the model's parameters we systematically simulated a number of proposed hypotheses of the mechanisms underlying post-flight orthostatic intolerance. The modeled hypotheses included hypovolemia, cardiac atrophy, increased leg venous compliance, decreased gain of the heart rate baroreflex, and a reduced ability to constrict venous and arterial smooth muscle. By simulating a tilt test response under these altered baseline conditions, we were able to compare the simulator's predictions to astronaut stand test data post-spaceflight. Our simulations indicate that although hypovolemia is the biggest single contributor, no single mechanism can account for the altered post-spaceflight heart rate dynamics. Rather, our simulations suggest that a superposition of reduced vasoconstriction of arterial and venous smooth muscle and hypovolemia can account for the dynamics of the heart rate response seen in astronauts post-flight (Figure 3).

The computational model was subsequently used to simulate the effects of a potential pharmacologic countermeasure, midodrine: an alpha agonist. We simulated the action of midodrine on a spaceflight adapted individual by increasing total peripheral resistance and decreasing unstressed venous volume by amounts compatible with experimental findings. The simulated post-flight heart rate response (Figure 4) of a midodrine-treated individual demonstrates a significant reduction in the excessive

tachycardia. The simulation suggests that the drug may have major potential benefit as a countermeasure for orthostatic intolerance, and human trials are warranted.

The computational model now has been implemented in JAVA, and is available for use by other investigators via the Web (**http://knut.kumoh.ac.kr/~mech/cvsim/Simulator.html**).

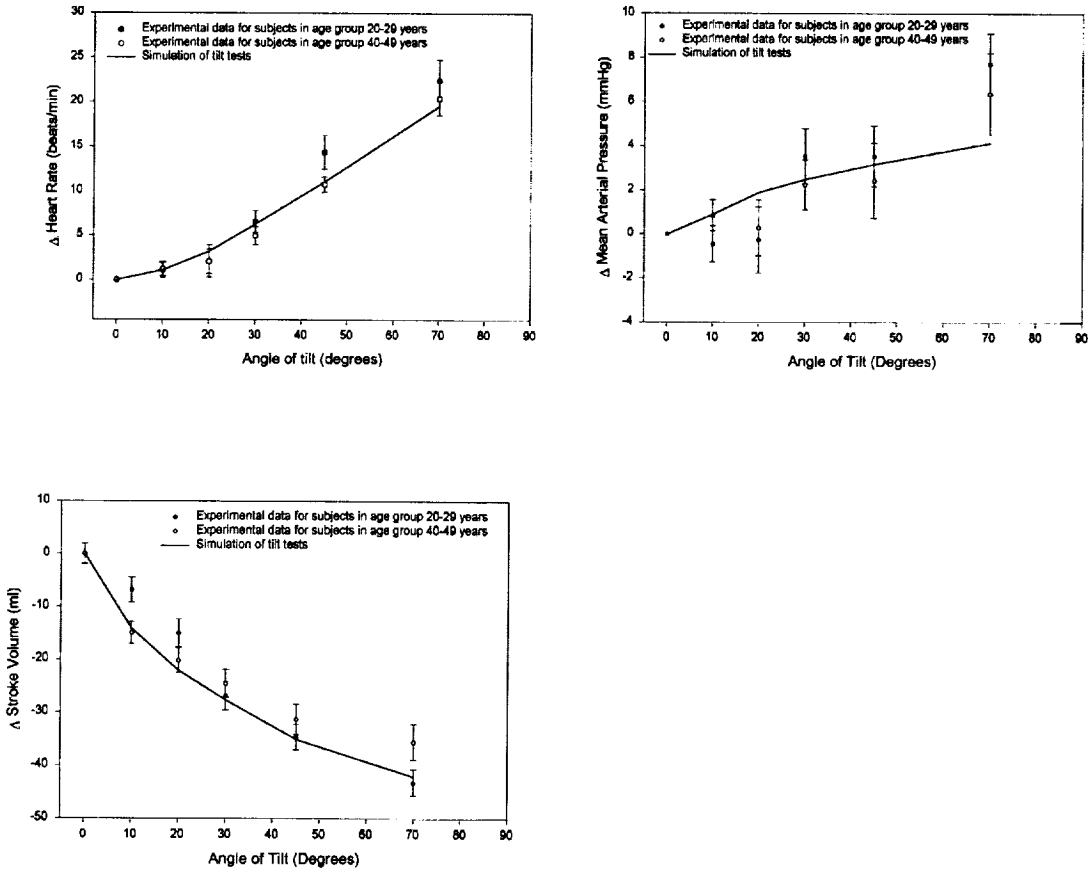


Figure 1: Hemodynamic response to tilt in subjects of different age groups (dots) and simulations (solid line)

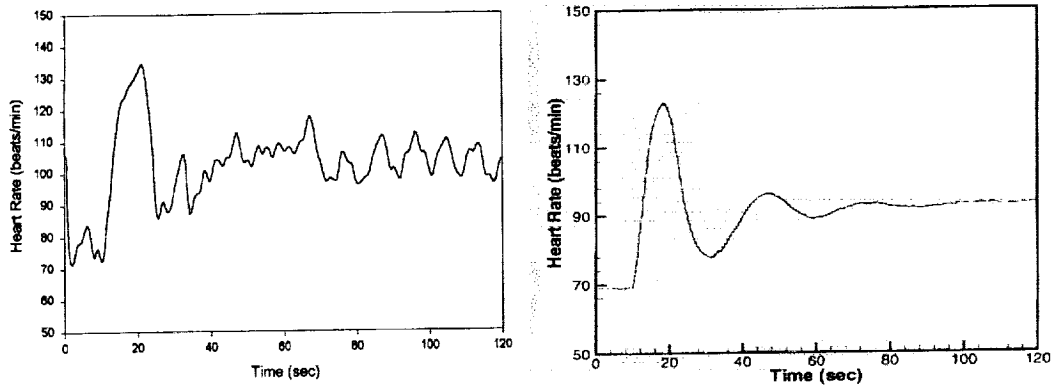


Figure 2: Heart rate response to standing in one astronaut pre-flight (left) and simulation of stand test (right).

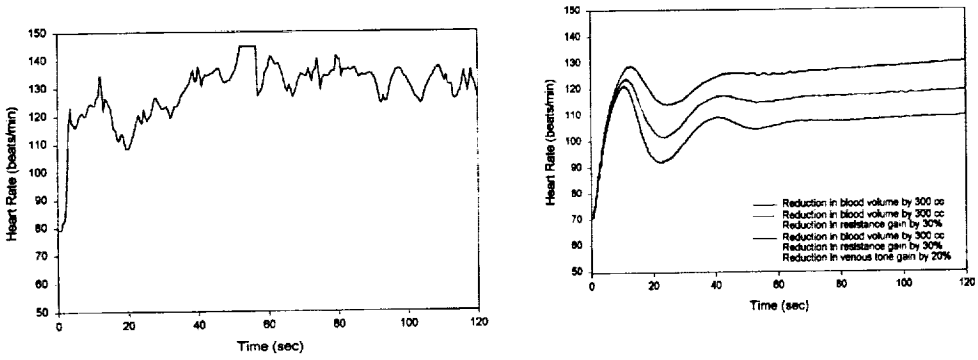


Figure 3: Heart rate response to standing on landing day (left) and superposition of simulation of various hypotheses (right).

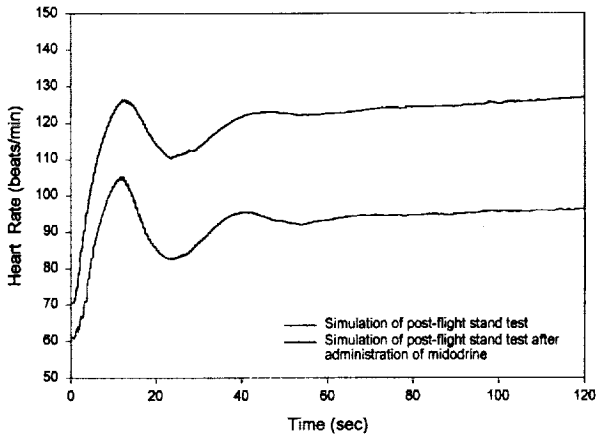


Figure 4: Simulation of post-flight stand test (black) and simulation of post-flight stand test after administration of midodrine.

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A. PROJECT RESEARCH ACTIVITY

In October 1997 a modest research program, *Computational Models of the Cardiovascular System and its Response to Microgravity*, was initiated in our laboratories with funding from the Cardiovascular Alterations Team of the National Space Biomedical Research Institute. The project has been highly productive, and a summary of our major accomplishments follows.

The objective of the first phase of the project was to develop a computational model capable of simulating the short term (transient) response of the cardiovascular system to abrupt gravitational challenges such as standing, head up tilt (HUT), or lower body negative pressure (LBNP) in normals or micro-gravity adapted individuals. The initial conditions and parameter assignments are chosen to represent the hypothesized state of the circulation at the time of the test (e.g. normal, volume-depleted, etc.). The model thus provides an intellectual framework with which to interpret experimental observations and to evaluate alternative physiologic hypotheses for the cause of orthostatic intolerance. Melchior [18] analyzed a number of previous models and summarized some of the general model requirements that are thought to be important in simulating the short-term response to orthostatic stress. Much like previously reported models [44, 53] our model is based on a closed loop lumped parameter hemodynamic model [41] with regional blood flow to major peripheral circulatory branches. Features such as non-linear regional venous compliances and venous valves have been implemented in accordance with previous work [31, 37, 53-55]. Capillary filtration of blood plasma into the interstitial fluid compartment, however, is an added feature of our model that has not previously been implemented for short-term studies [55, 56] and is of critical importance in simulating tilt and LBNP maneuvers. Blood pressure homeostasis is maintained in our model with the aid of the two major neural reflex control loops: the arterial baroreflex and the cardio-pulmonary reflex. Unlike older models [30, 31, 37, 50, 53-55] we put emphasis on separating sympathetic and parasympathetic reflex limbs. Furthermore, the four effector mechanisms (heart rate, cardiac contractility, regional peripheral resistance, and regional venous tone) have gain values which can be specified independently. Since autonomic dysfunction is believed to be at least in part responsible for orthostatic intolerance [57], the possibility of independently modifying the control of the heart and various vascular beds makes this model a powerful tool to investigate current hypotheses concerning the mechanisms underlying orthostatic intolerance.

The model is still evolving, and important features remain to be added, but in its present form it is already able to accurately represent the major features of the human response to orthostatic stress.

The Hemodynamic Model

Architecture

We have extended an earlier closed loop lumped parameter model of the cardiovascular system developed in our laboratory [40, 41] by accommodating blood flow to four major circulatory branches, an abdominal venous compartment, an inferior

and a superior thoracic vena cava (see Figure 1). The model thus consists of 12 compartments each of which is represented by a linear resistance and a capacitance that can be linear, non-linear, or time varying. The resulting model is mathematically described by a set of twelve coupled, linear, time-varying, first order differential equations which are implemented in C on a Linux operating system. A fourth-order adaptive step-size Runge Kutta integration routine is used to iteratively solve for the pressures from which flow rates and compartment volumes can be inferred. To initiate the integration, a set of initial pressures needs to be known. We estimate these pressures by solving a steady state problem equivalent to the circuit topology of figure 1. The resultant system of linear equations is solved using a modified Gauss-Jordan reduction algorithm to obtain the 12 initial pressures for a given set of parameter values [41].

The pumping action of the heart is represented by time-varying ventricular capacitances [41]. Atria are not represented; their function is, however, partially absorbed into the function of adjacent compartments. Diodes represent valves and ensure unidirectional flow through the ventricles and parts of the venous system. A pressure source, P_{th}, changes transmural pressure across the intra-thoracic compartments according to variations in pleural pressure. Pressure sources across the venous compartments in the abdomen, P_{bias-4}, the splanchnic area, P_{bias-3}, and the legs, P_{bias-1}, simulate changes in venous transmural pressure across these compartments due to postural changes or external lower body negative pressure.

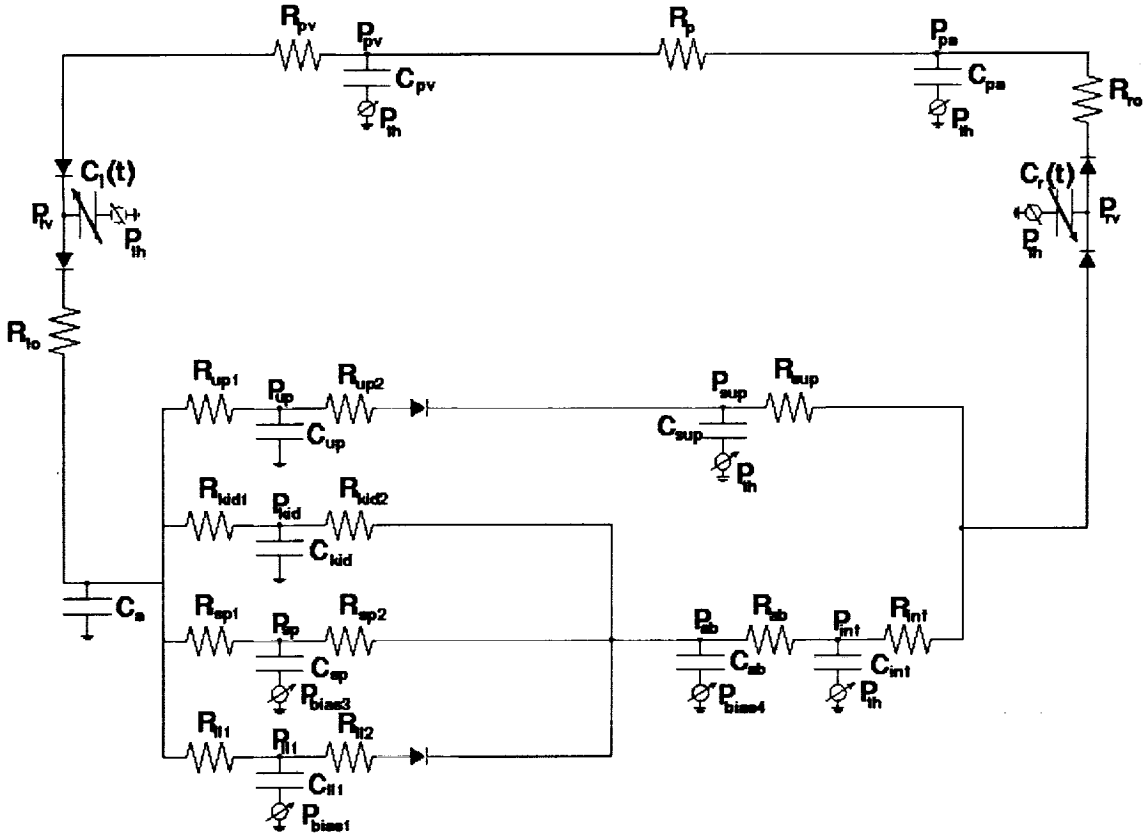


Figure 1. The Hemodynamic Model

In the supine posture, the physiologic range of venous transmural pressures is rather small and the venous pressure-volume relationship in the lower extremities and the intestinal veins can assumed to be linear. During high levels of LBNP or during quiet standing, however, the venous transmural pressures in the legs can increase to approximately 100 mmHg and the pressure-volume relationship of the dependent vasculature becomes markedly non-linear [58, 59]. We have therefore implemented non-linear compliances in all systemic compartments exposed to an external bias pressure. The functional form of the pressure-volume relationship in these compartments has been modeled on experimental data [60] in accordance with previously reported non-linear characteristics of the form [31, 55]:

$$\Delta V = \frac{2 \cdot \Delta V_{max}}{\pi} \cdot \arctan\left(\frac{\pi \cdot C_0}{2 \cdot \Delta V_{max}} \cdot \Delta P_{trans} \right)$$

where ΔV represents the change in compartment volume due to a change in transmural pressure, ΔP_{trans}. ΔV_{max} is the maximal change in compartment volume and C_0 represents the compartment compliance at baseline transmural pressure. Figure 2 shows the pressure volume relationships for the three non-linear compliances.

The interstitial fluid compartment is not explicitly modeled. Total blood volume is, however, modified as a function of time and the transmural pressure of a given compartment to simulate blood plasma sequestration into the interstitium.

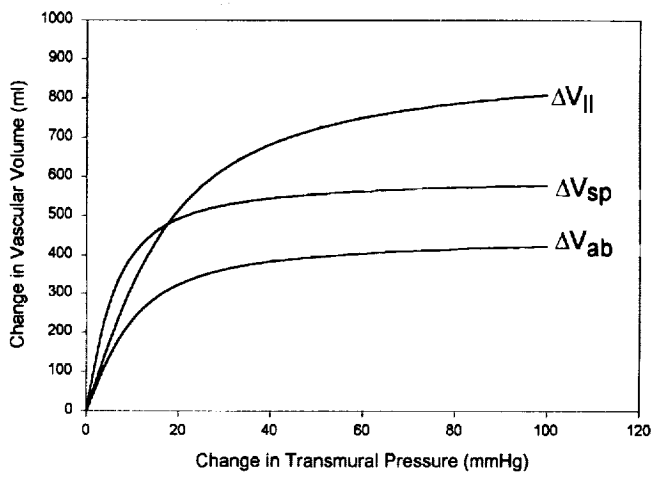


Figure 2. Pressure-volume relationships for the three non-linear venous compliances

Parameter Assignments

A detailed discussion of the rationale for assigning the parameter values for the hemodyamic model are provided in Appendix A.

The Reflex Model

Architecture

Our goal of simulating the transient hemodynamic behavior for up to 10 minutes after the onset of orthostatic stress can only be achieved with a sophisticated control model that includes neural and endocrine control loops, possibly adaptive changes in the control mechanisms, and autoregulation. We have thus far implemented the most important controls for short-term control – namely the two major neurally mediated cardiovascular reflexes: the arterial baroreflex and the cardiopulmonary reflex.

We have adopted and extended a previously reported regulatory set-point model of the arterial baroreflex that aims at maintaining mean arterial blood pressure constant by dynamically adjusting heart rate, peripheral resistance, venous zero pressure filling volume, and right and left end-systolic cardiac capacitances [32, 40, 41]. In addition to this representation of the arterial baroreflex, we have implemented a similar reflex loop to represent the cardiopulmonary reflex, which, at the moment only affects venous zero pressure filling volume and systemic arteriolar resistance. Briefly, locally sensed blood pressures are relayed to the autonomic nervous system (ANS) where error signals are generated by subtracting pre-defined set-points from the afferent pressure signals. These error signals subsequently dictate the efferent activity of the reflex model such that the error signals in the following computational steps approach zero (see Figure 3).

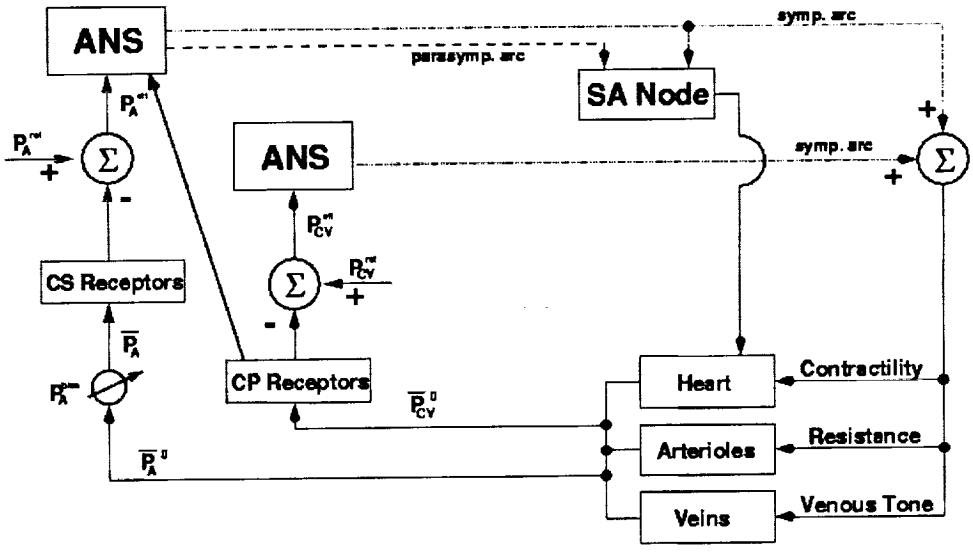


Figure 3. The reflex model

Threshold and Saturation

The input variables to the control system are mean arterial pressure, $\overline{P}_A$, for the arterial baroreflex and mean central venous pressure, $\overline{P}_{CV}$, for the cardiopulmonary reflex as substitutes for carotid sinus, $\overline{P}_{CS}$, and right atrial pressure, $\overline{P}_{AT}$, respectively. The

respective error signals are generated subtracting pre-defined set-points, $\overline{P}_A^{ref}$ and $\overline{P}_{CV}^{ref}$, from these input variables. The differences are non-linearly re-scaled by an arctangent of the form:

$$P_A^{eff}(t) = 18 \cdot \arctan\left(\frac{\overline{P}_A(t) - \overline{P}_A^{ref}}{18}\right)$$

to generate effective blood pressure deviations, $P_A^{eff}(t)$ and $P_{CV}^{eff}(t)$ [32]. These effective blood pressure deviations are thus limited to $\pm 18\pi/2$ mmHg ($\approx \pm$ 28 mmHg) and exhibit saturation at large deviations from the set-points as required [18].

Reflex Dynamics

To account for differences in timing of the reflex responses, the respective effective blood pressure deviations are weighted by impulse response functions which are characteristic of a parasympathetic, p(t), or sympathetic, s(t), response [61] (see Figure 4). The model thus provides for a rapid ($\approx$ 0.5 – 1 sec) parasympathetic reflex response and a slower acting ($\approx$ 10 – 15 sec) sympathetic reflex response. A 30 second running history of the effective pressures are used to compute instantaneous effector values by convolution with the appropriate effector specific impulse responses as the following example of RR-interval, I, feedback shows [41]:

$$I(t) = I_0 + \int_{k=0}^{k=30} (\alpha \cdot p(k) + \beta \cdot s(k)) \cdot P_A^{eff}(t-k)\, dk$$

The contribution of the arterial baroreflex to the instantaneous RR-interval at time t, I(t), is computed by adding a dynamically computed contribution to the baseline value, I_0. The impulse response functions, p(k) and s(k), are multiplied by the static gain values, α and β, for the respective reflex arc.

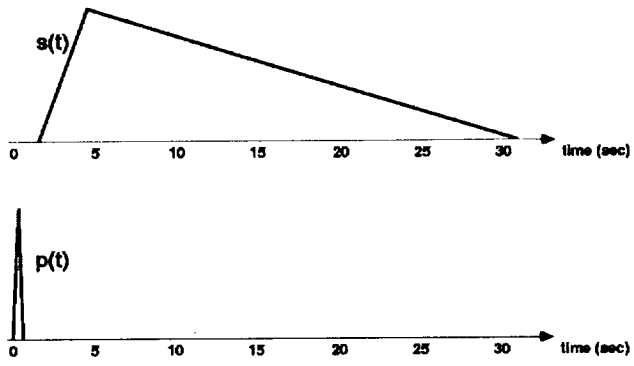


Figure 4. The impulse response functions of the sympathetic {s(t)} and parasympathetic {p(t)} systems

Gravitational Effects

During postural changes, the hydrostatic pressure at the carotid sinus receptor changes by an amount of $\rho \cdot g \cdot h \cdot \sin(\alpha)$, where h is the distance of the carotid sinus to the hydrostatic indifferent point (assumed to be the right atrium) and α denotes the angle of tilt of the carotid artery measured from the horizontal. To account for these effects, we have implemented a bias pressure source, P_A^{bias}, which modifies the sensed arterial pressure, $\overline{P}_A^0$, according to:

$$\overline{P}_A = \overline{P}_A^0 - P_A^{bias} \quad \text{and}$$
$$P_A^{bias} = \rho g h \sin(\alpha)$$

Our model does not currently include a separate aortic baroreceptor. It is important to recognize that while pressures detected at the carotid and aortic baroreceptors will be similar in the supine position and in microgravity, they will be quite different in acceleration or gravity fields. Our present model is not able to account for these differences, and adding this feature is planned for future work.

Cardiopulmonary and Baroreceptor Interaction

Finally, we have integrated into our model the modulation of the sensitivity of the arterial baroreflex by the cardiopulmonary reflex loop. It has been shown that central hypovolemia leads to an increase in heart rate gain of the arterial baroreflex [62]. Since the gain values for the RR-interval in our model are largely based on pharmacological studies as opposed to neck suction/pressure experiments, we had to re-scale the magnitude of the heart rate response to match our baseline gain values. We have therefore implemented the interaction in the form:

$$\text{Gain}_{\text{RR-interval}} = 20.0\frac{\text{ms}}{\text{mmHg}} - 0.7\frac{\text{ms}}{(\text{mmHg})^2} \cdot \overline{\text{P}}_{\text{CV}}$$

Parameter Assignments:

The rationale for assigning parameters to the reflex model is provided in Appendix A.

Simulation of Orthostatic Stress Tests

In the space life sciences, tilt table/stand tests and LBNP interventions are frequently used orthostatic stress tests. Both interventions have in common that increased transmural pressure across the dependent veins simulates a state of central hypovolemia and elicits a sequence of reflex responses.

Tilt table simulation

A tilt table intervention leads to rapid blood volume shifts from the thoracic to the dependent vascular beds. Also, the increased transmural pressure in the dependent vasculature leads to increased rates of blood plasma sequestration into the interstitium and thus a reduction in plasma volume [63, 64]. To simulate the rapid blood volume shift, the bias pressures, P_{bias-i}, across the splanchnic, lower limbs, and abdominal venous compartments were specified as a function of time:

$$P_{bias-i} = P_{max-i} \cdot \sin(\alpha(t))$$

where $\alpha(t)$ represents ramp in time from zero to the maximal angle of tilt. P_{max-i} denotes the maximal bias pressure across the respective compartment when upright posture is assumed. The slower reduction in blood plasma is modeled by appropriately modifying overall blood volume according to [63]:

$$V_{total} = 5300 + 400 \cdot 0.9^{t/60s}$$

Here t is the time after onset of tilt measured in seconds. Besides these hemodynamic changes, the elevation of the carotid sinus above the hydrostatic indifferent point leads to a reduction in sensed carotid sinus pressure by an amount of $\rho \cdot g \cdot h \cdot \sin(\alpha(t))$ (see section on reflex system).

LBNP Simulation

External pressure applied to the lower body is simulated by specifying the bias pressures across the lower body compartment, P_{bias-1}, according to:

$$P_{bias} = \begin{cases} 0 & before \\ \varepsilon P_{max} & after \end{cases} \text{ initiation of LBNP.}$$

Here P_{max} denotes the maximal external pressure applied to the lower limbs and ε is an "attenuation" factor that takes into account that not all the externally applied pressure is transmitted fully to all vessels in a given compartment. We chose $\varepsilon = 0.9$ for the lower limb in accordance with previously published findings [54]. In addition to blood pooling in the legs, significant pooling occurs in the pelvis and buttocks [65, 66]. In our model, the behavior of these two circulatory beds is partly represented by the abdominal venous compartment. Since the latter also represents the great abdominal veins, we simulate blood pooling in the pelvis and buttocks by applying a reduced bias pressure, $P_{bias-4} = \varepsilon \cdot P_{bias-1}$, to the abdominal venous compartment ($\varepsilon \approx 0.3$). The increased transmural hydrostatic pressure in the lower body and abdominal venous compartments leads to increased rates of fluid sequestration into the interstitium. The total amount of blood volume leaving the vascular system is a function of both level and duration of LBNP. At high levels of LBNP (-70 mm Hg to –75 mm Hg), it has been shown that a linear net

decrease of plasma volume on the order of 500 ml can be observed over the course of 10 min [67]. We simulate this plasma volume loss by reducing overall blood volume as a function of LBNP time, t, and LBNP level, P_{bias-1}, according to:

$$Blood\ Volume = 5700ml - \Delta V \cdot \frac{t}{600s} \text{ where}$$

$$\Delta V = 500ml \cdot \frac{P_{bias-1}}{70mmHg}$$

Model Verification

Steady State

Table 3 compares steady state values for certain hemodynamic parameters generated by the model to the range of normal values reported in the literature [68]. The simulations assume a total blood volume of 5700 ml, which is consistent with a 70 - 75 kg male subject having a body surface area of 1.7 to 2.1 m^2 [69, 70]. The table indicates excellent agreement with nominal hemodynamic values.

Table 3: Model Verification – Steady-State Results

Parameter	Units	Simulator Results	Normal Range
Cardiac Index	l/min/m^2	3.2	2.8 –4.2
Stroke Index	ml/beat/m^2	54	30 –65
Pressure	mmHg		
Left Ventricle			
Systolic		129	90 -140
End-diastolic		11	4 – 12
Systemic Arterial			
Systolic		128	90 – 140
Diastolic		79	60 – 90
Venae Cavae			
Maximum		4.5	2 –14
Minimum		3.0	0 – 8
Right Ventricle			
Systolic		30	15 – 28
Diastolic		13	5 – 16
Pulmonary Artery			
Systolic		30	15 – 28
Diastolic		13	5 – 16
PCWP			
Maximum		17	9 – 23
Minimum		11	1 – 12
Resistance	mmHg/ml/s		
Total systemic		1.0	0.68 – 1.05
Total pulmonary		0.12	0.11 – 0.19

Orthostatic Stress- Transient Response

Lower Body Negative Pressure

The model was tested against published data from LBNP experiments to assess its accuracy in representing the hemodynamic response to suddenly applied steps of different levels of LBNP each lasting for five minutes. Figure 5 shows our LBNP

simulation for various hemodynamic parameters together with experimental data from normal subjects [71]. Each experimental data point represents measurements made after 90 seconds of lower body negative pressure. Subjects were returned to atmospheric pressure for a 20-minute rest period between sequential applications of LBNP. The model predictions are plotted as solid lines, and the experimental points and standard deviations are superimposed.

There is an excellent match between simulation and experimental data for heart rate and stroke volume (and therefore cardiac output), but the blood pressure response (both systolic and diastolic) predicted by the model was somewhat lower than the experimental data, particularly at high levels of LBNP. The difference between simulation and experiment can potentially be explained by an insufficient resistance response in the model. Thus, although the major features of the model's response correlate very well with experimental data, additional tuning of the model will be required to improve the blood pressure response.

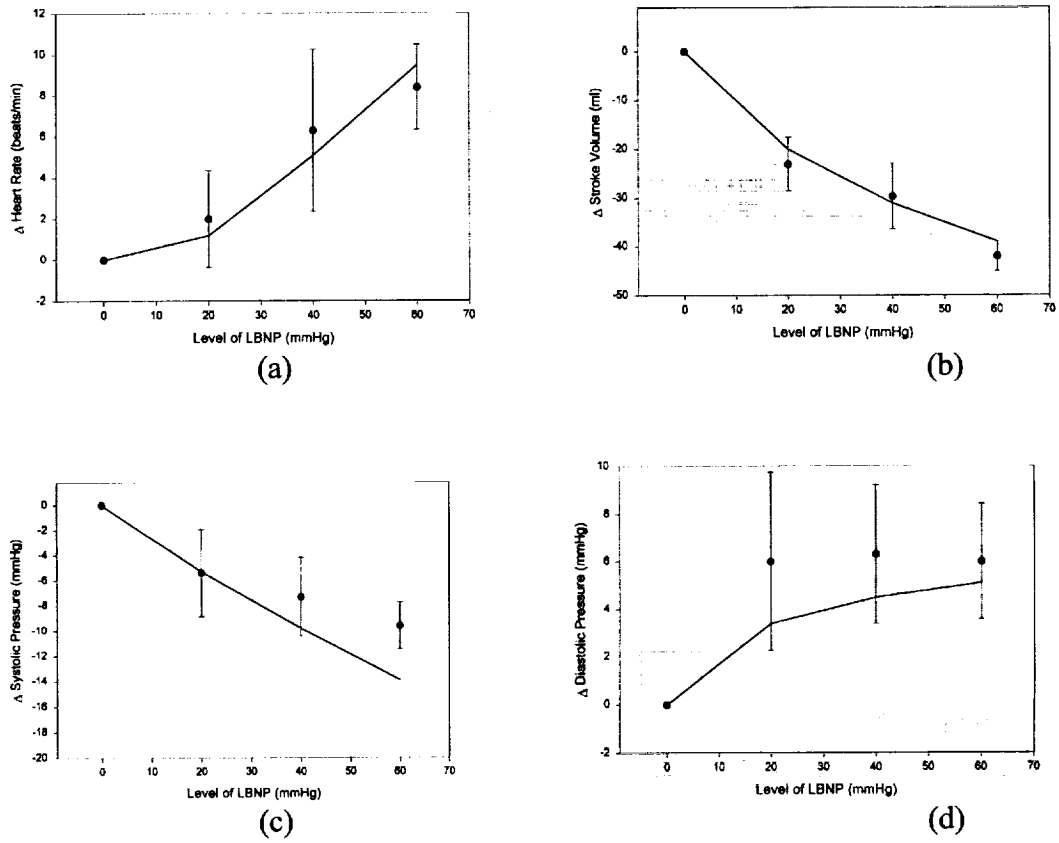


Figure 5. Comparison of LBNP simulation (solid lines) to experimental results: a) heart rate; b) stroke volume; c) systolic arterial pressure; d) diastolic arterial pressure.

Stand Test

Figure 6 shows a typical blood pressure and heart rate response to standing seen in astronauts pre-flight[72]. Blood pressure was recorded with a Finapress, which records arterial blood pressure at the tip of a finger. During the stand test, astronaut was asked to

hold the hand on which the pressure measurement was made at the level of the right atrium to minimize artifactual changes in pressure due to changes in the hydrostatic pressure component. The heart rate response was derived from an ECG recording.

In Figure 7, we depict the model's respective parameters during a stand test simulation, showing the model's phasic BP waveform. Although the model reproduces all major features of the short-term transient response to orthostatic stress quite well, there are minor discrepancies. The subject's blood pressure recording shows a critical damping after recovery from the orthostatic drop which the model is currently unable to reproduce. Furthermore, the model fails to reproduce the later (> 1min) oscillatory fluctuations (~ 6/min) in heart rate and blood pressure. This behavior was seen in most of the human data and will guide further enhancement of our reflex model. Overall, however, the time course and magnitude of the transient orthostatic drop (and recovery) in blood pressure, and the increase in heart rate match the data well.

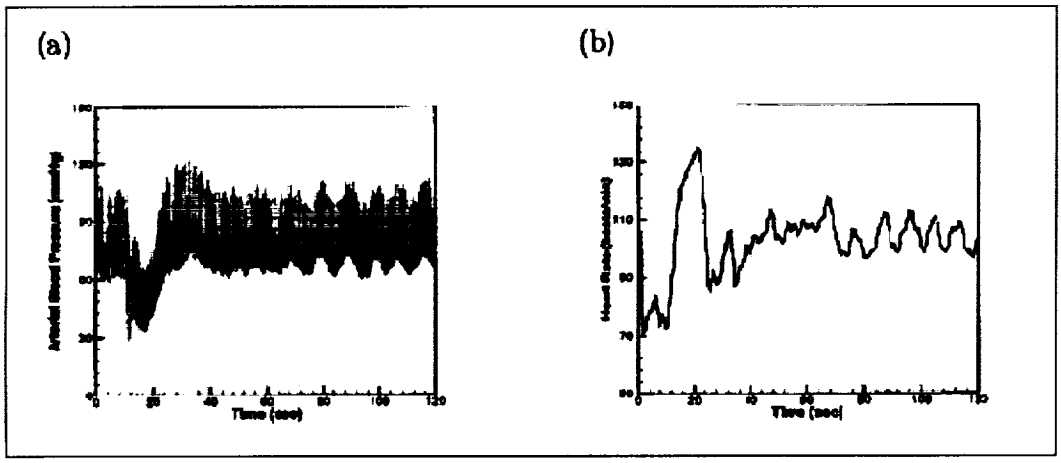


Figure 6. Stand test in pre-flight astronaut: a) arterial BP, b) heart rate.

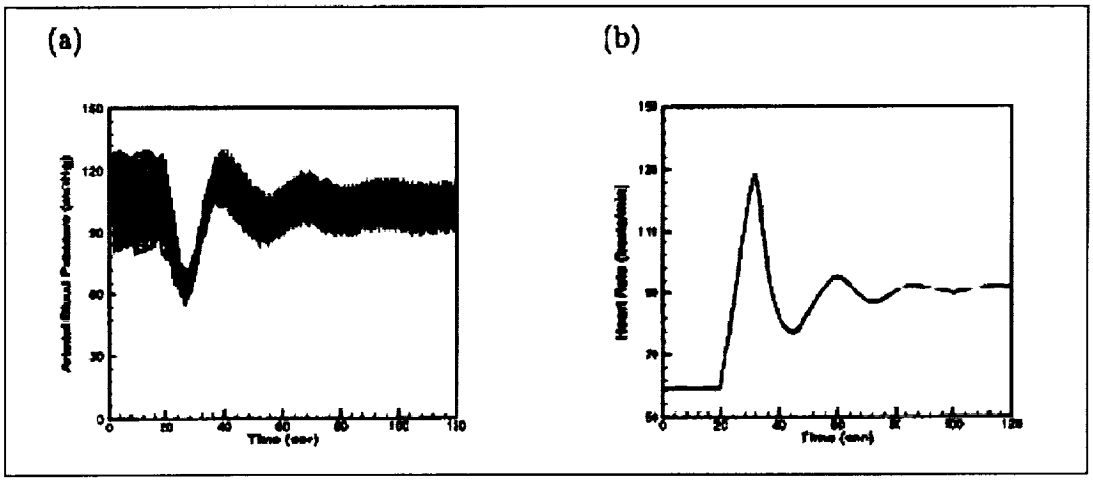


Figure 7. Simulated stand test: a) arterial BP, b) heart rate.

Tilt Test

We have been able to validate the model's predictions for the hemodynamic response to sudden tilts to different angles. Experimental data were obtained by Smith et. al. [73] who tilted subjects quickly to different angles from 10 to 70 degrees, each tilt lasting for 5 minutes. Five-minute recovery periods in the supine position were given between tilts. Figure 8 shows the simulated responses (solid line) and experimental data with standard deviations. The data presented are the averages for the last three minutes of each tilt angle. The simulated results match the experimental data quite closely, and confirm the model's general validity.

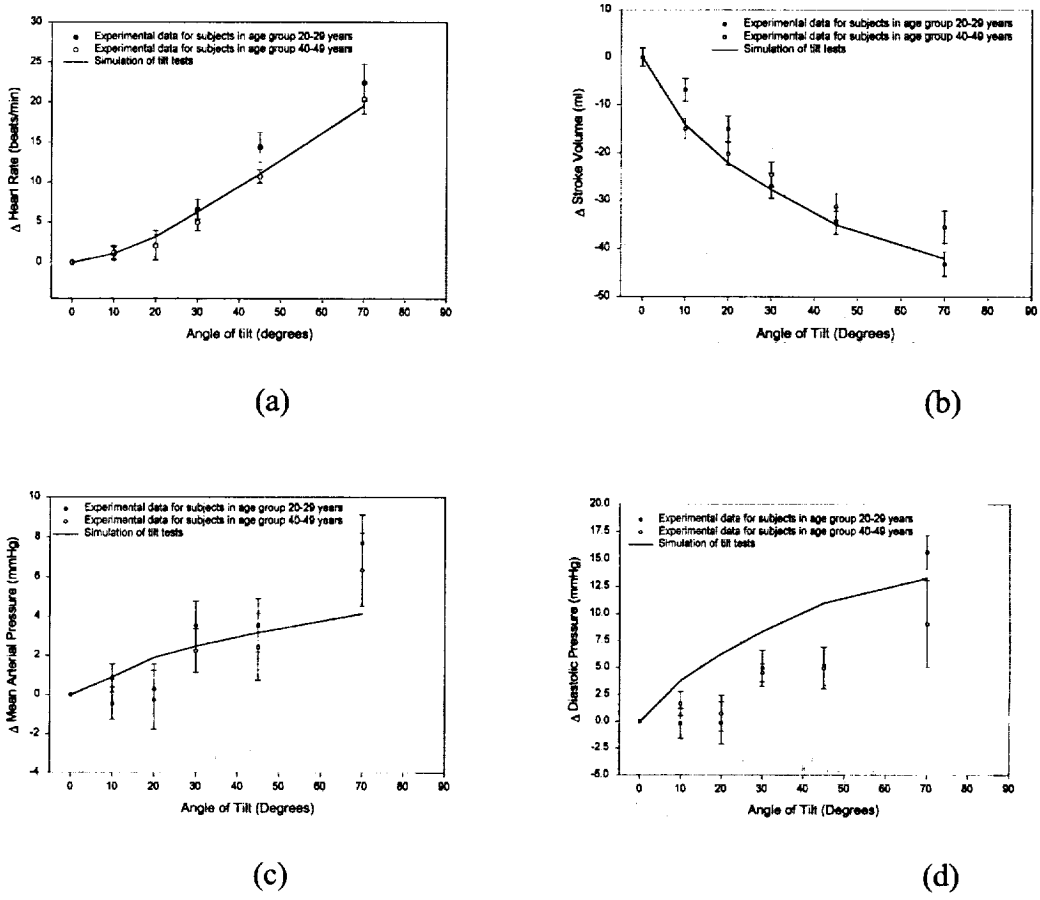


Figure 8. Comparison of tilt simulations to experimental results, showing changes in a) heart rate; b) stroke volume; c) mean arterial pressure; and d) diastolic arterial pressure.

Testing of Hypotheses

We have begun recently to use the model to explore hypotheses concerning the mechanisms underlying micro-gravity or bed-rest induced orthostatic intolerance. For example, we have been able to obtain pre- and post-flight experimental data from astronauts, through the assistance of Janice Fritsch-Yelle at Johnson Space Center [72]. Space flight induces significant changes in the stand-test results as demonstrated by comparing figure 9 (pre-flight) to figure 10 (post-flight). It is apparent that the post-flight blood pressure is maintained only by a drastic and continued increase in heart rate after standing.

We used the model to explore the predicted impact of a number of different hypotheses for OI. Comparing the simulated stand test transients of HR and ABP with the experimental data provides valuable insight into the feasibility of each hypothesis.

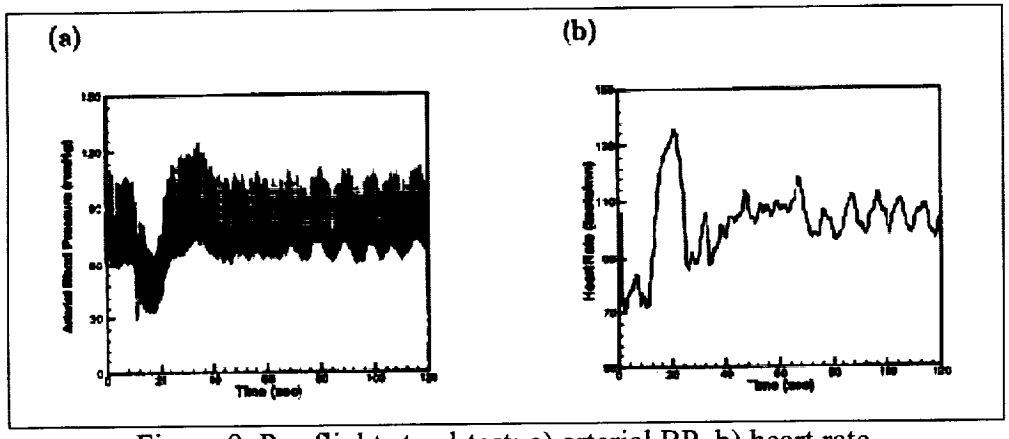


Figure 9. Pre-flight stand-test: a) arterial BP, b) heart rate.

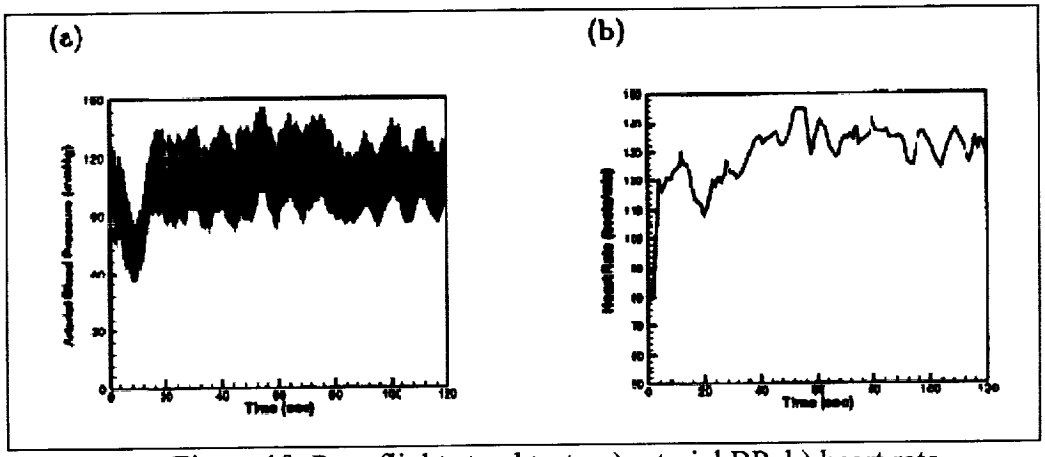


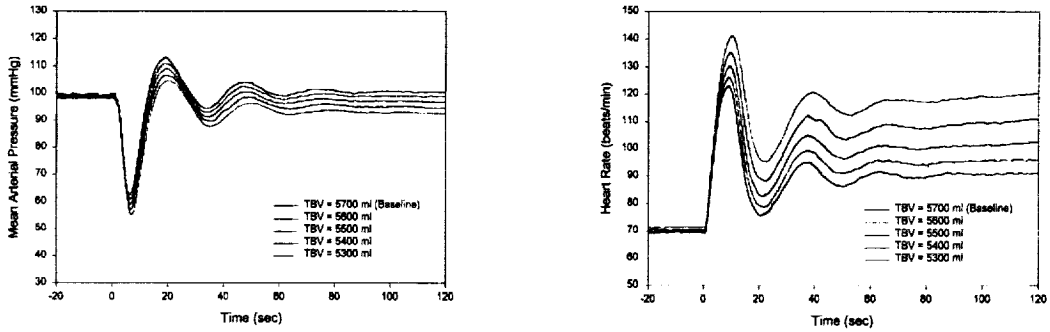
Figure 10. Post-flight stand test: a) arterial BP, b) heart rate.

We chose to simulate the following hypothetical 'what if' scenarios:

- 3% -13% decrease in plasma volume [74, 75]
- 5% - 20% reduction in parasympathetically-mediated heart rate gain of the arterial baroreflex [3]
- 5% - 20% increase in venous compliance of the legs [76]
- 5% - 20% cardiac atrophy[1][6, 77]
- 5% - 20% reduction in venous tone feedback gain [78]
- 5% - 20% reduction in resistance feedback gain[11-13, 16]

Figures 11 - 16 show the predicted heart rate and BP response to standing for simulations of each of the six hypotheses (Each graph includes the 'pre-flight' response for a baseline reference.)

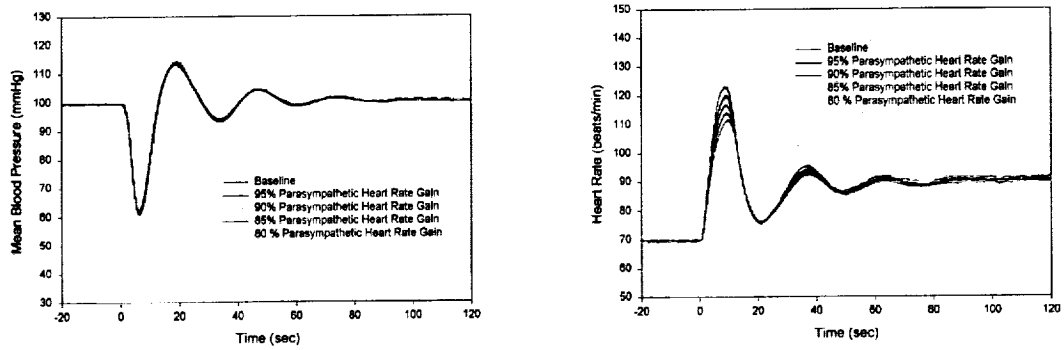
As can be seen, none of the proposed hypotheses when acting alone is capable of producing a heart rate response that is similar to the one seen after spaceflight in figure 10. In fact, it is quite remarkable how little impact on the overall response to orthostatic stress is made by individual interventions, with the exception of reduction in intravascular volume. Even a 400 cc blood volume reduction, however, fails to duplicate the experimental data of figure 9. However, if one hypothesizes that intravascular volume depletion s accompanied by a reduction in the reactivity of sympathetically mediated venous and arteriolar vasoconstriction, then the model predictions match very closely the experimental data (figure 16). Although we have not yet attempted to analyze the uniqueness of the simulation results, they are strongly suggestive that the etiology of OI is multi-factorial, and that strong candidates are the three factors modeled in figure 17. Obviously it is essential to invest major additional effort in exhaustive comparisons of simulations and human experimental data.



(a) (b)

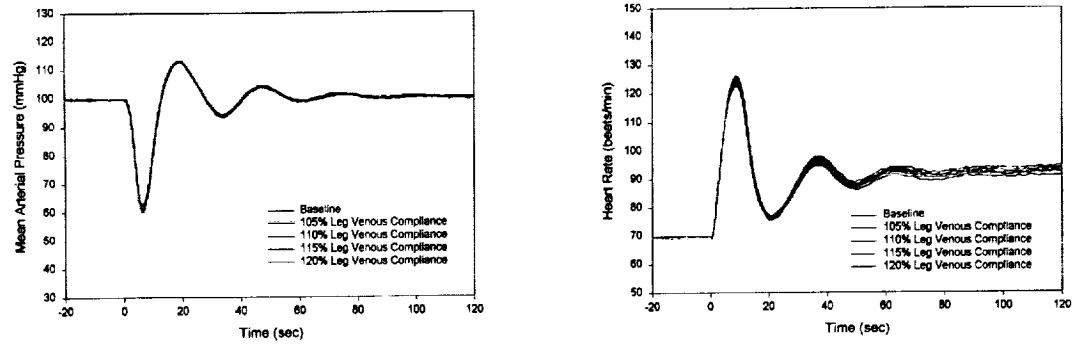
Figure 11: Mean BP (a), and heart rate (b) response to stand-test: decreases in intravascular volume

[1] To simulate the effects of a smaller and weaker heart we changed three cardiac parameters by the same percentage: 1) reduced the zero pressure filling volume, 2) reduced the end-diastolic compliance, and 3) increased the end-systolic compliance (decreased contractility).



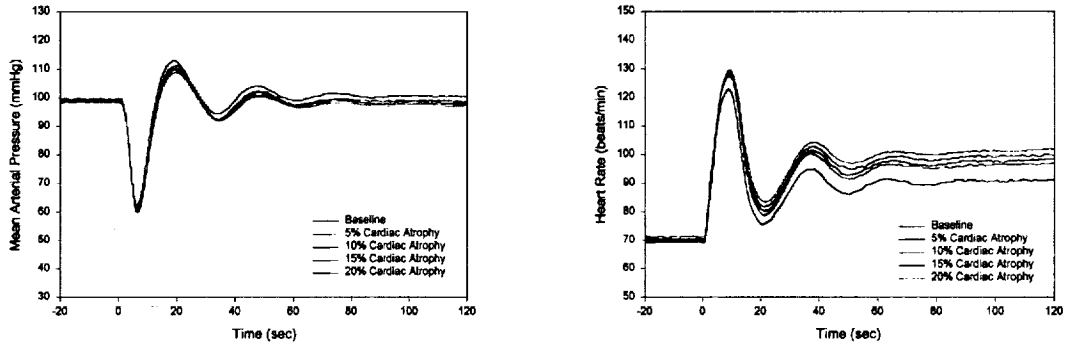
(a) (b)

Figure 12: Mean BP (a), and heart rate (b) response to stand-test: decreases in parasympathetic HR gain



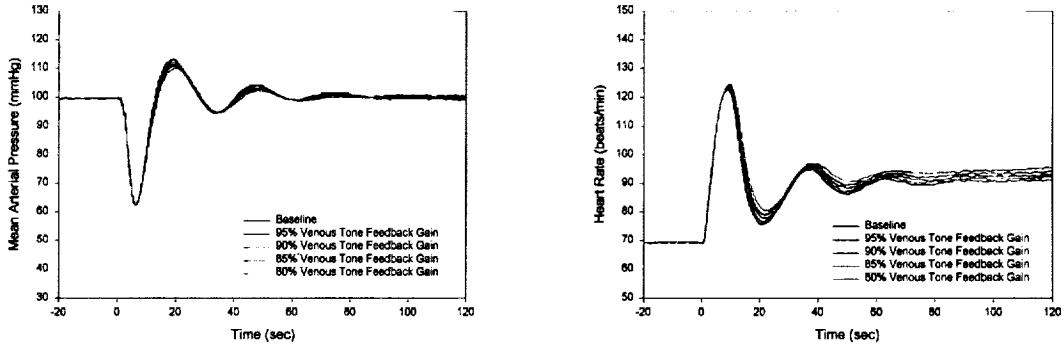
(a) (b)

Figure 13: Mean BP (a), and heart rate (b) response to stand-test: increases in leg venous compliance



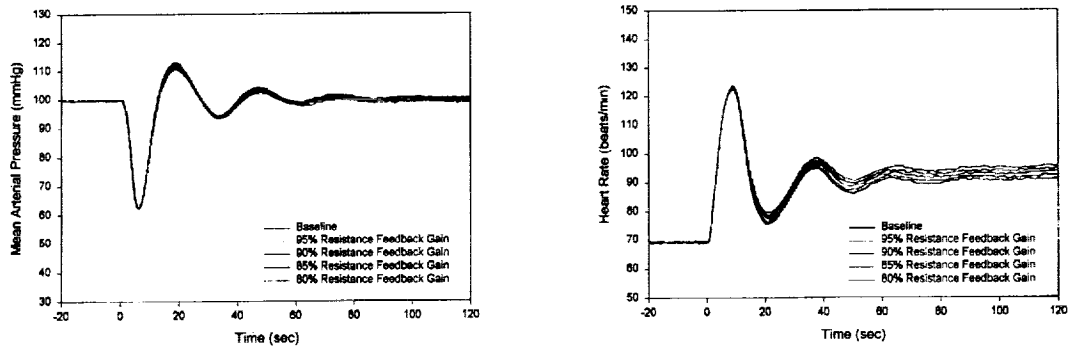
(a) (b)

Figure 14: Mean BP (a), and heart rate (b) response to stand-test: cardiac atrophy



(a) (b)

Figure 15: Mean BP (a), and heart rate (b) response to stand-test: decreases in venous tone gain



(a) (b)

Figure 16: Mean BP (a), and heart rate (b) response to stand-test: decreases in resistance gain.

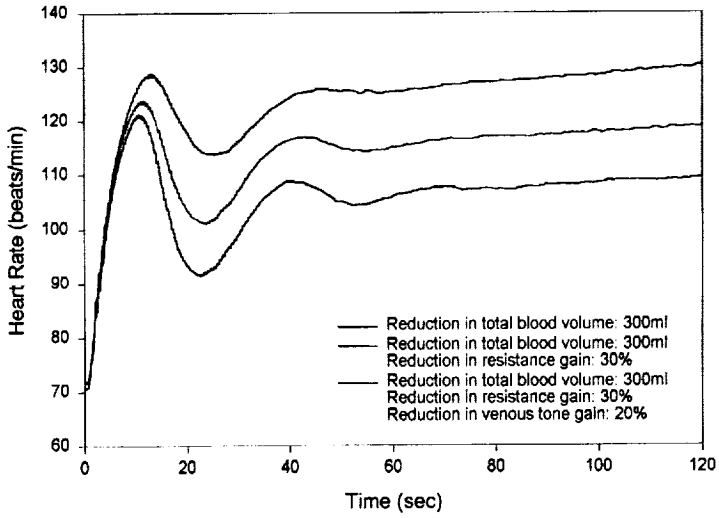


Figure 17. Simulated heart rate response to stand test with multiple interventions

Countermeasure Simulation

The model provides a powerful tool to explore the likely hemodynamic impact of different countermeasures. Since three of the more commonly accepted hypotheses for OI are a loss in intravascular volume, decreased venous constriction, and decreased arteriolar constriction during orthostatic stress, a logical proposed countermeasure is oral administration of midodrine, an alpha-adrenergic agonist. (This agent has shown promise

in the bed-rest studies currently under way in the cardiovascular team's research program.) In low doses, midodrine has been shown to reduce venous capacitance in humans [79] and in dogs [80] while leaving arterial blood pressure and blood flow constant. Venous occlusion plethysmography in humans revealed a decrease in leg pressure-circumference relation by about 5% after IV midodrine [79]. At higher doses midodrine has a strong effect on systemic vascular resistance. In humans up to a 60% increase in peripheral resistance has been documented [81]. We were unable to find documentation of an increase in alpha-receptor sensitivity with midodrine. Clinically, midodrine is useful in the management of orthostatic hypotension, although there is risk of inducing hypertension, particularly in the supine position.

We chose to simulate the actions of midodrine on a micro-gravity adapted cardiovascular system[2] by increasing the total peripheral resistance by 30% and decreasing the venous zero-pressure filling volume by 5%. We did not simulate any alteration in the gain of the effector feedback loops that had been decreased in microgravity. The simulated post-flight stand test results for an individual treated with midodrine are shown in figure 18, and suggest the drug has major potential benefit as a countermeasure for orthostatic intolerance.

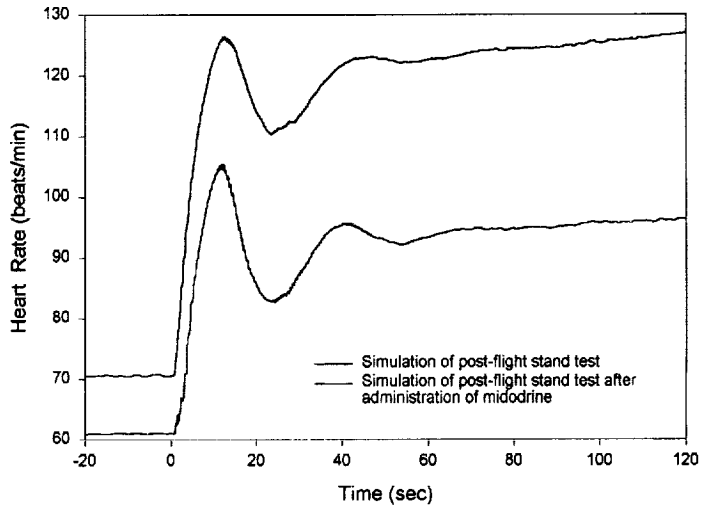


Figure 18. The simulated post-flight stand test results for an individual treated with midodrine

[2] Reductions in blood volume by 300 cc., resistance gain by 30%, and venous tone gain by 20% as demonstrated in figure 17.

Dissemination of the Model

The cardiovascular model being developed in this project is of great potential value to other NASA - affiliated investigators, who may wish to perform their own "what if ?" simulations. We have made significant progress in developing a version of the simulator in JAVA that will be freely available via the Internet. This work has been primarily the contribution of Prof. Eun Bo Shim and his students in Korea. The preliminary version of the software may be accessed at:
http://knut.kumoh.ac.kr/~mech/cvsim/Simulator.html

The model is also available on our NIH-supported research archive of physiological signals and software at **www.physionet.org** [82].

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

As a result of the progress made in this project during these past three years, we are now in an excellent position to study the nature of an astronaut's cardiovascular response to a change in gravitational environment. The tools thus far developed address the first 5-10 minutes after onset of gravitational stress upon return to the normal gravitational environment.

These models are now available to all members of the cardiovascular team. They are intended to be used in the interpretation of new results from physiologic experiments and to design new testing protocols to test and refine existing hypotheses regarding the physiologic changes that occur during prolonged space flight. The completed studies presented in the previous section demonstrate the utility of the model and prove its worth in the continuing effort to develop effective countermeasures.

Our studies to date have highlighted intravascular volume depletion accompanied by a reduction in the reactivity of sympathetically mediated venous and arteriolar vasoconstriction as a potential explanation of the conditions leading up to syncope. They have also provided a demonstration of how midodrine can reverse these factors, suggesting an effective countermeasure consistent with the results from the bedrest studies. It is important to recognize that with a system as complex as this, it is not possible to rely on one's intuition alone to envision all of the possible implications of a particular intervention. The model, however, to the extent that it captures the essence of the physiologic response, extends our capabilities and thereby becomes indispensable.

In the next phase of research, it will be necessary to extend and refine the model in two ways. First, the capability needs to be developed to make the model subject-specific. That is, we need to be able to identify the parameter values of a specific individual so that the model can be used to predict the needs for countermeasures or interventions of that individual. Person-to-person variability is such that an appropriate countermeasure for one astronaut may be very different from that required by another. Second, while the first 5-10 minutes are critical, we also need to extend the capabilities of the model to simulate long-term behavior. This can be done in stages, first incorporating the hormonal response, but eventually encompassing the entire range of possible adaptive responses that lead to orthostatic intolerance in the first place.

The synergistic effect between experimental studies and modeling efforts cannot be overemphasized. Models provide a rational framework with which to interpret clinical results and guide future research. This is especially true in the light of small subject numbers which is a chronic problem in the space life science research program. One major implication of the current work to future space biomedical research is its demonstration that experimental studies can greatly benefit from concurrent modeling studies and, vice versa, modeling studies rely on good experimental data to verify their output.

The computational model developed in this research project is also of great potential value in clinical medicine. Intensive care units (ICUs) provide continuous and invasive measurements of respiratory and hemodynamic status in acutely ill patients. Such monitoring permits the early detection of changes in the patient's condition and provides information that both directs therapy and assists in evaluating the response to

treatment. The ICU medical staff must re-assess these patients frequently on the basis of data from clinical observations, bedside monitors, mechanical ventilators and a wide variety of lab tests. The responsibilities of ICU staffs are complex [89-91]; they usually assess more than 50 measurements, laboratory values, and physical findings, etc. In addition, most of the current medical monitoring systems include built-in alarms, and permit users to change the alarm limits. However, these automated alarms operate independently, and are so unaware of the clinical context that they have a high rate of false alarms. ICU staff may delay their response to alarms that are frequently in error, or even ignore them [89]. Providing life support in the ICU is becoming an increasingly complex task as the volume of monitoring data increases. Improvement in the organization and interpretation of the clinical data will have the potential of increasing quality and efficiency in the ICU.

This large amount of monitored ICU data has created "information overload", which leads to errors and mishaps in ICU care. The great responsibilities of ICU staff and high performance expectations can lead to both emotional and physical fatigue. There are occasional tragedies, most of which are due to human error, reported in ICU care. Abramson [92] estimated that about 90 of 150 adverse occurrences in a surgical ICU were caused by human error. Similar results regarding human errors occurring in general ICU care were reported in [90]. As monitoring technology advances, more and more clinical data will be presented to ICU staff. Therefore, information overload will become an increasingly serious problem unless better methods are developed to organize and present the data to ICU staff.

About 20 years ago, investigators began to apply expert system techniques to the problem of interpreting ICU data, beginning in the domain of respiratory physiology and ventilators [93]. In intensive care areas, automated methods that monitor the hemodynamic status of patients must reason about and respond to changes in large amounts of clinical data that are both numeric (such as that arterial blood pressure is 150/50) and symbolic (such as the interpretation of an echocardiogram). Intelligent monitoring systems must handle both types of data. Combining the technology of expert systems with mathematical models offers a unique approach to the problem.

Several years ago we reported the design and implementation of a knowledge-based expert system to interpret ICU data [94, 95]. The system was designed around a simple version of the cardiovascular model. The question was whether it was possible to identify a set of model parameters that would generate hemodynamic data similar to that observed from the patient. We were successful in demonstrating that the model parameters could indeed be identified using a search strategy guided by the knowledge-based expert system. In that work, however, we made use of *simulated* patient data, not real-world clinical data. A powerful ground-based spin-off is therefore the integration of a cardiovascular simulator into an intelligent patient monitoring system that allows for parameter estimation in real-time.

A second ground-based application of the cardiovascular simulator is its use as a powerful environment for teaching of normal and abnormal cardiovascular physiology. When confronted with a complex physiologic system such as the cardiovascular system, students cannot be expected to gain an intuitive understanding of the interdependence of all parameters and variables. Provided with a simulator of cardiovascular function,

however, students can develop intuition by simulating various scenarios such as hemorrhage, cardiac atrophy or hypertrophy and observe the effects of such a change to the overall dynamics of the system. By checking their own predictions with the simulator's output, they are able to check their intuition and learn about the system's behavior.

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APPENDIX A: Project Research Data and Modeling Parameters

I. The hemodynamic model

Where possible, parameter values for the hemodynamic model are based on the literature as indicated in Table 1. However, in some cases, values had to be estimated, as is the case with the regional systemic resistance values.

Table 1. Parameter Values for the Hemodynamic Model

Compartment	ZPFV		Compliance		Inflow Resistance		Outflow Resistance	
	ml	Ref.	ml/mmHg	Ref.	PRU	Ref.	PRU	Ref.
Right Ventricle	15	[41]	1.2 - 20	[41]	0.012[1]		0.003[2]	
Pulm. Arteries	90	[41]	4.3	[83]	0.003	[84]		
Pulm. Veins	490	[83]	8.4	[83]	0.08[4]	[85]		
Left Ventricle	15	[41]	0.4 - 10	[41]	0.01	[86]	0.006[3]	
Syst. Arteries	715	[41]	2.0	[83]	0.006	[86]		
Systemic Veins								
Upper Body	650	[83]	8		3.9[5]	estimate	0.23	[83]
Kidney	150	estimate	15	estimate	4.1[5]	estimate	0.3	
Splanchnic	1300	[87]	55	[87]	3.0[5]	estimate	0.18	[83]
Lower Limbs	350	[83]	16	[88]	3.9[5]	estimate		
Abdominal Veins	250	[53]	25	[53]				
Inferior Vena Cava	75	[53]	2	[53]	0.01		0.015	[83]
Superior Vena Cava	10	[53]	15	[53]	0.28		0.060	[83]

Notes:
1. RV inflow is the parallel combination of the inferior and superior vena cavae outflows
2. RV outflow resistance is the same as the pulmonary artery inflow resistance.
3. LV outflow resistance is the same as the aortic inflow resistance.
4. This is the pulmonary vascular resistance.
5. This is the resistance of the microcirculation of this portion of the systemic circulation.

Resistances

Based on estimates of the percent of cardiac output to the four circulatory branches (upper body 23%, kidney 22%, splanchnic circulation 30%, and lower body 25%) we modeled arteriolar resistance values to produce a total peripheral resistance of 1.0 PRU (peripheral resistance units – mmHg·s/ml). This method generates a splanchnic arteriolar resistance of 3.0 PRU, which is similar to values previously used in other cardiovascular models [87]. The resistance values on the venous side of the circulation are largely taken from Beneken and DeWitt [83] (see Table 1). We assumed that resistance to venous flow in the kidneys is of the same order of magnitude as that found in the legs. Although the pulmonary vascular resistance is known to be dependent on cardiac output and exhibits non-linear characteristics, it was approximated by a constant

value [41, 85]. Outflow resistances of the ventricles have previously been estimated [86]. We have adopted the values used by Davis [41]. The left ventricular inflow resistance is also based on estimates by Davis [41].

Capacitances

In addition to being a function of transmural pressure [89], the aortic capacitance has been documented to change dramatically with age [90]. For our purposes, however, it is sufficient to choose the lumped arterial compliance such that the time constant for blood flow from the arterial to the venous side of the systemic circulation is reproduced. Measurements of the time constant for arterio-venous blood flow have determined an exponential time constant of 1.9s [91]. This value is adequately reproduced using a lumped arterial compliance of 2.0 ml/mmHg. The capacitance value for the head and arm veins (upper body compartment) have been taken from Beneken and DeWitt [83]. The capacitances of the splanchnic and kidney compartments have been chosen to reproduce 60 ml/mmHg in accordance with previous models [87]. We attribute 55 ml/mmHg to the compliance of the intestines, liver, and the spleen and 5 ml/mmHg to the kidneys. The leg compartments have been assigned a combined venous capacitance of 19 ml/mmHg [88]. Anatomically, C_{lll} represents the compliance of both lower extremities up to the inguinal ligament. It has been assigned a capacitance of 16 ml/mmHg [60, 92]. We have modeled the pressure-volume relationships for the abdominal, inferior thoracic, and superior thoracic venae cavae on previous models of the cardiovascular system [31]. The pulmonary compliances have been taken from Beneken and DeWitt [83].

The lower limb, splanchnic, and abdominal venous compartments all exhibit non-linear pressure-volume relations according to equation 1.1. The compliances discussed here are the compliances at normal supine transmural pressures (C_0 in equation 1.1). During orthostatic stress, transmural pressures increase in the dependent vascular beds and the respective compliances are computed by differentiating equation 1.1 with respect to ΔP.

Pumping Chambers

Both ventricles are characterized by time dependent compliances that vary between a minimum end-systolic and a maximum diastolic value according to a predefined functional form. Estimates of left ventricular maximum diastolic compliance, $C_{l,diast}$, are frequently based on estimates of left ventricular end-diastolic elastance, $E_{l,diast}$ ($E_{l,diast} = 1/C_{l,diast}$). The latter have been reported to range between 0.15 mmHg/ml to 0.6 mmHg/ml for normal subjects [93]. If directly translated into $C_{l,diast}$, these values would suggest a maximum diastolic left ventricular capacitance of 1.7 to 6.7 ml/mmHg. This method, however, underestimates $C_{l,diast}$. Due to the curvilinear diastolic pressure-volume relationship, the maximum capacitance occurs during early diastole before the ventricle is filled to near maximum capacity. Previous estimates of maximum diastolic capacitance of 10 ml/mmHg for the left ventricle [41] seem compatible with experimental observations [6]. The right ventricular maximum diastolic capacitance, $C_{r,diast}$, is approximately twice the left ventricular value [94]. The left ventricular end-systolic elastance, $E_{l,sys}$, has been reported in 15 normal subjects to be 2.0±0.7 mmHg/ml [95]. If translated into $C_{l,sys}$, a value close to 0.5 ml/mmHg seems reasonable. The right ventricular end-systolic capacitance, $C_{r,sys}$, has been shown to range from 0.35 to 1.6

ml/mmHg [94]. It was chosen such that the pressure generated by the right ventricle is approximately 1/3 of the pressure generated by the left ventricle [41]. The pumping action of the ventricles is realized by varying the ventricular capacitances between $C_{r,diast}$ and $C_{r,sys}$ and $C_{l,diast}$ and $C_{l,sys}$ according to a time-varying elastance model [41, 95]. During systole, the right and left ventricular elastances, $E_r(t)$ and $E_l(t)$, change from their respective minimum diastolic value to their end-systolic value according to an inverted half cosine function. During early diastolic relaxation, they change from their respective end-systolic values to their minimum diastolic value according to a half cosine function. During the remaining diastolic time, the elastances stay at their minimum diastolic value [41]. The ventricular compliances are computed according to $C(t) = 1/E(t)$. Figure A1 depicts the left ventricular elastance as a function of time, and compares it with experiment [95].

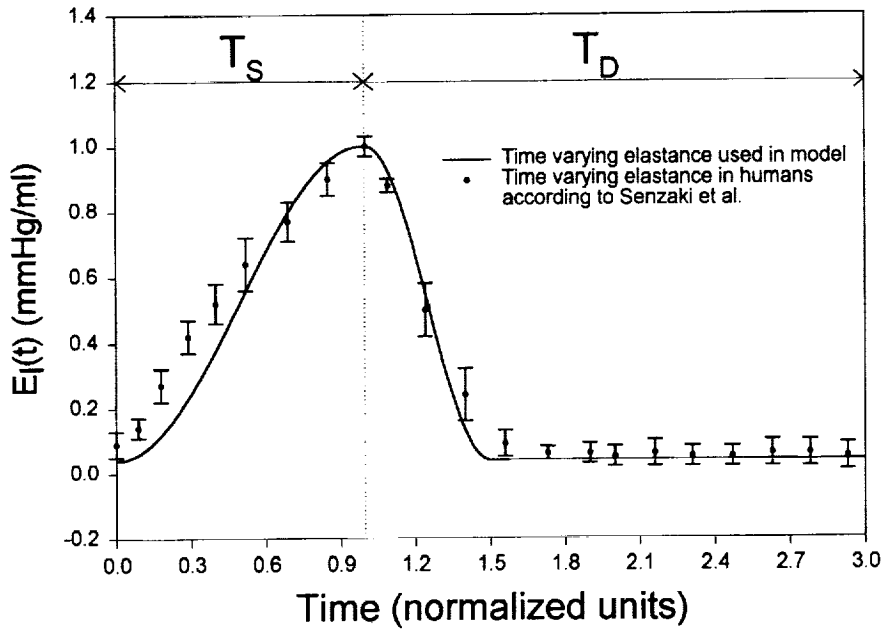


Figure A1: Left ventricular elastance vs. time.

Volume

Studies of total blood volume have report values of 75-80 ml/kg body weight for normal male subjects [70, 96]. We have set total blood volume to 5700 ml, which simulates a 71-75 kg normal male subject.

The distribution of blood volume within the circulation is tabulated in standard physiology textbooks [97]. Approximately 15% of blood is found in the aorta, systemic arteries, and arterioles, 69% is found in the capillaries, venules, and systemic veins, 9% is found in the pulmonary circulation, and 7% is found in the heart. Assuming an unstressed arterial volume of 715 ml, the systemic arterial tree contains roughly 900 ml when stressed to a mean arterial pressure of 92 mmHg (715 ml + 2 ml/mmHg · 92 mmHg),

which is about 15% of total blood volume. Assuming right and left ventricular filling pressures of 5 mmHg and 10 mmHg, respectively, and 15 ml unstressed volumes for each ventricle, the total cardiac volume at end of diastole is 230 ml. This is only about 4% of total blood volume, which is partly due to the lack of atria in our hemodynamic model. Furthermore, there seems to be considerable variation in cardiopulmonary blood volume as shown by Levinson et al. who reported that cardiopulmonary blood volume ranged from 301 to 546 ml/m^2 of body surface area [98]. We have adopted Davis' pulmonary unstressed volumes of 90 ml and 490 ml for the pulmonary arteries and veins, respectively [41]. This distribution of arterial and cardiopulmonary blood volumes allocates approximately 3600 ml, or 63% of total blood volume, to the capillaries and systemic venous circulation.

The unstressed volumes of the upper body and lower limbs were largely modeled on previously published estimates [83]. The unstressed volumes for the splanchnic venous and central venous compartments were guided by previously published models [31, 87].

I. The Reflex Model

This section of the appendix provides details concerning the assignment of parameter values to the control model, including references to the literature.

Reflex latencies

Several factors contribute to the time delay between baroreflex stimulation and effector organ response: afferent nerve time response, central nervous processing, efferent transmission, and effector organ response. Borst and Karemaker [99] reported a 0.55 second delay for heart rate response to electrical stimulation of the carotid sinus nerve. They also noted a 2-3 second delay for changes in diastolic pressure, which they attributed to reflex changes in peripheral resistance. Berger and co-workers characterized the canine heart rate response to sympathetic and parasympathetic stimulation and report a reflex latency of approximately 1.7 seconds for the sympathetic reflex limb [61]. Currently, we use a 0.5 second delay for the parasympathetic reflex response and 2.0 seconds for all sympathetic reflex arcs.

Static gain values

Baroreflex

Table 2 documents the baroreflex gain values incorporated into the present version of the model. Experimental techniques that have been used to characterize the baroreflex **heart rate gain** or RR-interval gain include pharmacological interventions, application of neck suction/neck pressure devices, and transfer function analysis. We have adopted DeBoer's static gain values of 9 ms/mmHg for β-sympathetic feedback and 9 ms/mmHg for parasympathetic feedback [32]. We incorporated Davis' model of **contractility** feedback, which states that, under maximal stimulation, the arterial baroreflex can alter end-systolic left and right ventricular cardiac elastances by a factor of 2 [41]. We followed Davis' determination of the **peripheral resistance gain** of the arterial baroreflex [41]. **Venous tone feedback** changes are difficult to quantify in

humans. Dog experiments revealed a maximum change of systemic reservoir volume of approximately 12 ml/kg under maximal carotid sinus stimulation [100], 6-7 ml/kg of which have been shown to originate from the abdominal vessels [101]. If scaled to represent a 75 kg human, 12 ml/kg maximal deviation in reservoir volume would suggest a maximal zero pressure volume deviation of 900ml. Given the limitation of the afferent pressure signal of our arterial baroreflex model to ±28 mmHg, 900 ml maximum volume deviation would translate into a static gain value for venous tone feedback of approximately 31 ml/mmHg.

Table 2. Baroreflex Gain Values

Reflex Limb	Units	**Gain Value**	**Timing**	**Reference**
R-R Interval	ms/mmHg	9.0	Parasymp.	[32]
		9.0	Symp.	[32]
Contractility	$ml/mmHg^2$			
LV End-systolic		-0.007	Symp.	[41]
RV End-systolic		-0.021	Symp.	[41]
Resistance	PRU/mmHg			
Upper Body		-0.01	Symp.	[41]
Kidney		-0.01	Symp.	[41]
Splanchnic		-0.01	Symp.	[41]
Lower limb		-0.01	Symp.	[41]
Venous Tone (ZPFV)	ml/mmHg			
Upper Body		5.3		[100]
Kidney		1.3		[100]
Splanchnic		13.3		[100]
Lower limb		6.7		[100]

Cardiopulmonary Reflex

Table 3 documents the gain values for the cardiopulmonary reflex. Experiments in humans have shown splanchnic blood volume to change by approximately 500 ml in response to a 1000 ml hemorrhage without significant changes in heart rate, cardiac output, and arteriolar resistance [102]. Assuming this response to be mediated by the cardiopulmonary reflex only, we can get a rough estimate of the cardiopulmonary reflex gain to venous tone by assuming that the contribution of the splanchnic circulation is about 60% of the total venoconstriction response. These assumptions would suggest a static venous tone feedback gain of approximately 100 ml/mmHg, as the cardiopulmonary afferent pressure signal is limited to ±8 mmHg. The effects of the cardiopulmonary reflex on peripheral resistance can be estimated form LBNP experiments. Low level of LBNP usually elicits a vasoconstrictor response without increases in heart rate [62, 103]. Calculating resistance as cardiac output/(mean arterial pressure – central venous pressure), the data presented by Pawelczyk and Raven [62] suggest a static gain of the cardiopulmonary arteriolar reflex limb of 0.05-0.06 PRU/mmHg.

Table 3. Cardiopulmonary Reflex Gain Values

Reflex Limb	**Units**	**Gain Value**	**Timing**	**Reference**
Resistance	**PRU/mmHg**			
Upper body		-0.06	Symp.	[62]
Kidney		-0.06	Symp.	[62]
Splanchnic		-0.06	Symp.	[62]
Lower limb		-0.06	Symp.	[62]
Venous Tone (ZPFV)	**ml/mmHg**			
Upper body		13.5	Symp.	[102]
Kidney		2.7	Symp.	[102]
Splanchnic		54.0	Symp.	[102]
Lower limb		19.8	Symp.	[102]

APPENDIX B: Publications Supported through NSBRI

Shim, E.B., Kamm, R.D., Heldt, T., Mark, R.G. Computational Modeling of Cardiovascular System after the Fontan Procedure. Proceedings of the 1999 Bioengineering Conference ASME 1999, BED-Vol. 42, 15-16, 1999.

Heldt, T., Shim, E.B., Kamm, R.D., Mark, R.G. Computational Models of Cardiovascular Function for Analysis of Post-Flight Orthostatic Intolerance. Computers in Cardiology 1999; 26:213-21.

Heldt, T. Shim, E.B., Kamm, R.D., Mark, R.G. Computational Model of Cardiovascular Function during Orthostatic Stress. Accepted to: Computers in Cardiology 2000.

Shim, E.B., Kamm, R.D. Numerical Analysis of the Blood Flow Through a Stenosed Artery Using a Multiscale Simulation Method. Accepted to: Computers in Cardiology 2000.

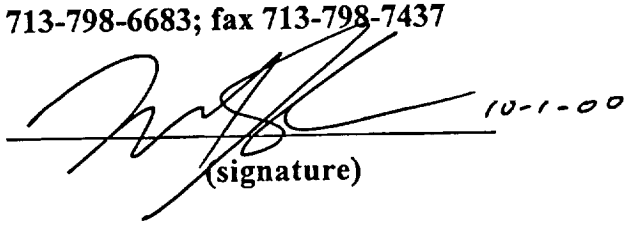
Shim, E.B., Kamm, R.D., Heldt, T.H., Mark, R.G. Computational Modeling of the Cardiovascular System after the Fontan Procedure. To be submitted to: J. Biomech. Engineering.

Heldt, T., Shim, E.B., Kamm, R.D., Mark, R.G. Computational Model of Cardiovascular Function for Simulation of Gravitational Stress. To be submited to: J. Appl. Physiol.

NSBRI CARDIOVASCULAR TEAM

PROJECT TITLE: **Cardiac Atrophy**

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EXECUTIVE SUMMARY

Key findings and discoveries:

1. The requisite physiological and molecular methods were implemented to assess cardiac function in mouse and rat hearts in response to cardiac loading and unloading, including M-mode and Doppler echocardiographic assessment of cardiac structure and function in mice, and in vivo left ventricular hemodynamic recordings.
2. Programmed cell death (apoptosis) in cardiac myocytes was demonstrated in response to altered load. Multiple complementary approaches to monitor and alleviate apoptosis were investigated.
3. Contractility is impaired in mouse myocytes with down-regulation of SERCA2, a gene whose expression is defective, following decreased loading.
4. Heterotopic transplantation, the best-established model of cardiac unloading, was implented successfully. Unloading by this means was shown to induce cardiac atrophy, down-regulation of SERCA2, and up-regulation of nominal markers of hypertrophy (despite the opposite effect on growth).
5. Contractile reserve was depressed in myocytes from unloaded hearts. Impaired contractile reserve in myocytes from unloaded hearts is related to the inability to augment intracellular systolic Ca_{2+} during this challenge.
6. Growth hormone is a potential countermeasure, which partials rescues SERCA2 expression during cardiac atrophy, but higher doses than tested will be required to correct the defects in contractile reserve and cardiac mass.
7. Biochemical studies to identify novel load-regulated proteins, for mechanistic insights and as potential sites for intervention, led to two fundamental discoveries. Load regulates activation of RNA polymerase II, which controls global rates of RNA synthesis per cell, via the protein kinase, Cdk7. Load also regulates the

mitogen-activated protein kinase, TAK1, which mediates (in part) the effects of load on cell survival and gene expression.

8. Genetic studies to identify load-regulated genes, for mechanistic insights and as potential sites for intervention, led to the discovery of more than 50 differentially expressed genes, beyond those that have been reported previously to be targets of load, including many signaling proteins: Rap1B, protein phosphatase 1γ [PP1γ], inhibitor protein phosphatase 2A [IPP2A], mss4, dynamin-like protein 1 [DLP-1], and the putative mechanosensor, ILK.

Satisfaction of the hypotheses, technology, objectives and aims:

The cardiovascular system undergoes multiple changes during prolonged space-flight as adaptation to the microgravity environment. During spaceflight, the cardiovascular system is not subjected to the biomechanical stresses associated with changes in posture in a gravitational field. Space-flight is associated with a modest reduction in intravascular volume and red blood cell mass, a decrease in arterial blood pressure, and a relative shift of intravascular volume from the lower body to the thorax and head; importantly, these adaptations occur even in the presence of regular exercise regimens and hydration during space-flight missions. It is recognized that the integrated cardiovascular adaptation to prolonged spaceflight may be in the net beneficial in the microgravity environment, but may be maladaptive when the cardiovascular system is subjected to severe abrupt stresses such as reentry into a higher gravitational field or the requirement to perform sustained and near-maximal exercise. In the present grant period, the NSBRI Cardiovascular Alterations team addressed three potential critical risks that may be imposed during long duration spaceflight. Each of these potential critical risks requires the elucidation of mechanisms in order to develop rational and effective countermeasures that can be applied in human long-duration spaceflight. The critical risks include: (1) the development of orthostatic hypotension and risk of syncope upon reentry into the earth (and potentially Mars) gravitational field; (2) the susceptibility to rhythm disturbances; and (3) the reduction in cardiac mass.

Our team focused specifically on the problem of changes in cardiac remodeling and gene expression which occur in response to cardiac unloading (cardiac atrophy) in rodent models, and compared these observations with changes that occur in response to excess load (cardiac hypertrophy). The observations in this grant period support our index hypothesis that the plasticity of the heart to adapt to perturbations in load is limited, and that cardiac unloading stimulates changes in gene expression which are phenotypic of cardiac hypertrophy. In short, directionally similar changes in gene expression occur during BOTH increases and decreases in cardiac muscle cell size, indicating these are reflective of cell remodeling per se.

This paradigm shift has important predictive implications for future hypothesis-testing and for specific counter-measures, as a number of adverse pathways affecting contractility, cardiac compliance, and even cell survival are activated as part of the known hypertrophic gene program.

The significance of this work, directed at cardiac atrophy, was highlighted by preliminary human data, made known during the study period by Dr. J. Yelle, Head of the Cardiovascular Laboratory of the Johnson Space Center. Whereas no reduction in LV mass was found using echocardiography, after short-duration NASA missions (n=13), a significant, reproducible, 10% loss of LV mass was seen in long-duration MIR missions (n=4). This compels greater diligence, in monitoring the long-term effects of microgravity

on cardiac mass in astronauts, and also reinforces the scientific need to understand the mechanisms and molecular details, underlying this gross change.

Thus, the overall hypotheses posed by the investigators have gained substantial reinforcement from both animal and human studies.

Implications for risk reduction related to the Critical Research Path and Earth medical problems:

The whole-animal and isolated-cell studies together demonstrate, unambiguously, loss of cardiac mass, alterations of normal gene expression, and impaired contractile reserve. Thus, further studies of cardiac atrophy during unloading are clearly warranted.

Growth hormone can partially rescue impaired expression of SERCA2, encoding the calcium "pump," in this model of cardiac atrophy. Thus, further studies of growth hormone are clearly warranted, at higher dosages and in concert with other countermeasures.

Mechanical load affects numerous targets that had never previously been identified but were disclosed by novel biochemical and genetic methods. Thus, future work is needed to explore the functional contribution of these candidate effectors, to develop countermeasures for those, like TAK1, whose consequences are adverse, and, conversely, to develop therapies based on those, like Cdk7, whose effects on cardiac mass or function is shown to be beneficial.

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I. Project Research Activity

A. Establishing the requisite procedures to assess cardiac function in mouse and rat hearts in response to cardiac loading and unloading.

1) Echocardiographic assessment of cardiac structure and function in mice. A central experimental strategy in our project has been the measurement of changes in function and gene expression in hearts with cardiac unloading in comparison with hypertrophied hearts subjected to excess load. As a test-bed for physiologic measurements in normal and heterotopic transplanted small rat and mouse hearts, we first adapted our model of ascending aortic stenosis (AS) from the rat to the mouse (FVB/n strain). Our longitudinal studies in this model are now published (Ding et al. 1999; Ding et al. 2000). This microsurgical procedure reporducibly causes an initial concentric hypertrophy (studied after 4-wks AS), transition to early heart failure 8-wks after banding, and progression by severe failure 13-wks after banding. We have demonstrated the feasibility of performing serial echocardiographic assessments of cardiac function and structure using two-dimensional targeted M-mode echocardiography in this model, as illustrated below. By 7-wks AS, LV weight is markedly increased relative to body weight (5.7 ± 0.4 vs 2.7 ± 0.1 mg/g, $p<0.05$) and left ventricular fractional shortening is diminished by echocardiography (endocardial: $48\pm7\%$ vs $54\pm5\%$, $p<0.05$; midwall: $24\pm4\%$ vs $32\pm5\%$m $p<0.05$). In collaboration with the Beth Israel Deaconess Medical Center Cardiac Mouse Imaging Facility, work is underway to adapt this noninvasive approach to the unloaded heart of similar small size (heterotropic cardiac transplantation in the rat).

2) In vivo left ventricular hemodynamic recordings. We also successfully transferred our technology for high fidelity hemodynamic measurements by left ventricular catheterization from rat to small mouse hearts. In normal mice, the left ventricle is catheterized via the left carotid approach where direct apical puncture is used in mice with ascending aortic stenosis. In vivo LV pressure recordings demonstrated the marked elevation of LV systolic pressure in banded mice, during early compensatory hypertrophy, followed by the depression of LV pressure generation which is characteristic of heart failure. In addition, the increased LVEDP with a distinct A wave was detected which is characteristic of severe hypertrophy.

3) Doppler measures of aortic and mitral flow velocities in mice. As a complementary non-invasive approach, we utilized Doppler assessment of aortic and mitral flow velocities. These measurements have been serially performed in aortic stenosis mice. As another example, cardiac hypertrophy was also triggered using a transgene encoding an activated form of the serine/threonine kinase, TAK1, which has been implicated in signal transduction for transforming growth factor beta and other cytokines that are upregulated in hypertrophied hearts: a 50% reduction in the velocity of LV inflow and LV outflow was detected, consistent with diastolic and systolic dysfunction.

B. Assessment of left ventricular cardiomyocyte apoptosis in situ.

In this grant period, developed and published complementary approaches for the assessment of cardiomyocyte apoptosis in situ (Ding et al.2000). In this publication, we demonstrate that cardiomyocyte apoptosis develops in mice during sustained biomechanical load due to experimental aortic stenosis. The studied showed that the development of apoptosis is time-dependent during perturbation of load, and is not detected in early compensatory hypertrophy or age-matched controls.

Rationale: These analytical approaches will be used in the next grant period to test the hypothesis that apoptosis is induced by cardiac unloading during simulated microgravity: observations in unloaded hearts (heteretopic transplantation) will be compared with hypertrophied hearts subjected to excess load.

Results: Quantitative assessment of left ventricular in situ myocyte apoptosis using confocal microscopy and Tunel staining was done by Dr. Lorell, in collaboration with Dr. Borg and Dr. Price (Consultants on this application). Left ventricular myocyte apoptosis is virtually absent in sham-operated control mice at all ages, and in 4-week aortic stenosis mice at the stage of adaptive hypertrophy. However, left ventricular myocyte apoptosis is observed in all 8-week aortic stenosis mice with a low prevalence of ~2 per 1000 myocytes. DNA fragmentation consistent with myocyte apoptosis was confirmed by the electrophoretic DNA laddering method. This frequency of Tunel-positive myocytes is remarkably similar to that reported in other models of hypertrophy at the stage of early failure, such as transgenic mice with Gsα overexpression (Geng et al. 1999). Comparable studies using an array of complementary criteria were performed by Dr. Schneider, in both genetic and pathophysiological settings.

Interpretation: The technique of in situ nick-end labeling (Tunel) has the potential to identify DNA fragmentation due to necrosis or rapid DNA repair (Didenko et al. 1996; Kanoh et al. 1999). Therefore, in collaboration with Dr. Vladimir Didenko, we confirmed that Tunel-positive myocyte nuclei in the model also show in situ ligation of single-base 3' overhangs. This highly specific approach distinquishes apoptotic nuclei from cells with nonapoptotic DNA damage (Didenko et al. 1996). We also developed biochemical measurement of caspase-3 activation in organ tissue samples. In our hands, this biochemical method readily detects caspase-3 activation in thymus undergoing apoptosis (a tissue used in the laboratory as positive-control for tissue apoptosis); in contrast, the low frequency of apoptosis in the hypertrophied heart is below the limits of reproducible detection by this biochemical approach. To address this, we have recently adapted the approach reported by Narula et al. in which release of mitochrondrial cytochrome C into the cytosol is identified by electron microscopy using immunogold labeling with anti-cytochrome C antibodies (Narula et al. 1999). This approach is poorly suited for quantitation of the frequency of in situ myocyte apoptosis in large populations of cells from multiple samples. However, this approach, which detects an early step in the apoptotic pathway (release of mitochondrial cytochrome C responsible for activation of the proteolytic caspase cascade) provides a corroborative methodology for detection of myocyte apoptosis independent of the detection of DNA fragmentation.

C. Contractility is impaired in mouse myocytes with down-regulation of SERCA2.

In this grant period, contractile reserve was measured in isolated adult ventricular mouse myocytes from normal, 4-wk aortic stenosis mice, and 8-wk aortic stenosis mice at stage of early heart failure. This work, which was presented as abstract at the 1999 American Heart Association Scientific Sessions (Ito et al. 1999), is under invited revision (Circulation Research).

Rationale: These studies are important to this project for two reasons. First, we are now successfully applying this experimental approach in the mouse to heterotopic transplanted rat hearts of similar small size using aortic cannulation and collagenase perfusion to obtain Ca^{2+} tolerant ventricular myocytes. Second, as discussed below, we have demonstrated that unloaded hearts respond with the downregulation of SERCA2.

The aortic stenosis model provides a test-bed for comparison of the functional role of SERCA2 in loaded and unloaded hearts.

Results: Intracellular calibrated Ca_{2+} transients and cell contraction were measured in myocytes loaded with the fluorescent indicator, fluo-3, using the fluorescence microscopy and high speed camera-video motion system in Dr. Lorell's laboratory. Under basal conditions (pacing frequency 0.5 Hz and perfusate Ca_{2+} 1.2 mmol/l), the amplitude of contraction and systolic and diastolic intracellular Ca_{2+} levels were comparable in all groups. However, striking differences were observed when the myocytes "were put to work". Contractile reserve was measured in two different ways: (1) the abrupt and stepped elevation of extracellular Ca_{2+} with a rapid solution switcher at constant pacing rate, and (2) stepped increases in pacing frequency at constant extracellular Ca_{2+} concentration. In response to both stimuli, the normal and 4-wk AS myocytes showed a similar increase in the amplitude of shortening and the amplitude of the Ca_{2+} transients, as well as acceleration of its kinetics. Thus, changes in cardiomyocyte size per se are not accompanied by a depression in contractile reserve. However, myocytes from 8-wk AS mice with depressed expression of SERCA2 showed severe depression of contractile reserve in response to increases in pacing frequency as well as increases in extracellular Ca_{2+} . These experiments demonstrate that impaired contractile reserve is mechanistically related to the failure to augment systolic Ca_{2+}. Studies are underway to measure sarcoplasmic reticulum Ca_{2+} content under basal and stimulated conditions using two different and complementary approaches: measurement of Na/Ca exchange current triggered by caffeine, and measurement of the abrupt increase in intracellular Ca_{2+} detected by fluo-3 fluorescence following application of caffeine with a rapid solution switcher.

Interpretation: These ongoing studies suggest that impaired frequency-dependent contractile reserve in the myocytes with reduced SERCA2 expression is related to the failure to increase sarcoplasmic reticulum Ca_{2+} stores during rapid pacing. In addition, the downregulation of SERCA2 is associated with depressed contractile reserve in isolated myocytes at the 8-wk stage of hypertrophy when the reduction in left ventricular ejection fraction and fractional shortening is very modest; in contrast, contractile reserve is normal in 4-wk hypertrophied myocytes in which SERCA2 expression is still preserved. Myosin composition (increased ratio of § to α-myosin heavy chain) is altered at both stages and cannot explain the differences in contractile reserve. Transgenic experiments provide an approach to prove a causal relationship between SERCA2 expression and depressed contractile reserve. In collaboration with Dr. Wolfgang Dillman of the University of San Diego, we have completed experiments which show that the defective frequency-dependent contractile reserve in AS mice and myocytes can be partially rescued in vivo, and at level of isolated myocytes, by chronic transgenic overexpression of SERCA2a. These experiments were presented in part at the 1999 American Heart Association Scientific Sessions (Ito et al. 1999), and are under preparation as manuscript to be submitted to Nature Medicine.

D. Development of the model of cardiac unloading: heterotopic transplantation.

In this grant period, the NSBRI Cardiovascular Alterations Team projects did not have a mandate to perform space-flown experiments in animal models or humans within the initial RFA. Therefore, we have studied the well-established model of cardiac unloading by heterotopic transplantation of the heart to the abdomen in isogenic receipients. This model provides a test-bed for examination of both progressive degrees of

unloading and cardiac atrophy, as well as testing of "proof-of-concept" using a moderately severe magnitude of atrophy. The aim here is to generate observations and potential countermeasures which can later be rigorously and rapidly tested when opportunites become available for animal experimentation and human physiologic studies in long-duration spaceflown missions. This model also provides synergy with other NSBRI Cardiovascular Alterations Team projects which are examining: 1) baroreceptor function and cardiac deconditioning in human subjects exposed to short-term bedrest; and 2) vascular responses in rats subjected to mild cardiac unloading by tail-suspension. We have successfully implemented models of heterotopic cardiac transplantation to the abdominal aorta in rats (Dr. Lorell's laboratory) and mice (Dr. Schneider's laboratory). In the present grant period, we predominantly focused on the rat model because of reguired synergy for comparison with other rat models on the existing NSBRI teams. An advantage of the model, scientifically and economically, is the availability of the in situ heart (the control) and unloaded transplanted heart for paired analysis. Pilot experiments showed no difference in cardiac mass or morphology between in situ native hearts following transplantation of an isogenic heart, and hearts from age-matched animals undergoing sham operation. Because we have shown unequivocal effects of gender in our published experiments of excess cardiac load in rodents (Weinberg et al.1999; Douglas et al. 1997), we deliberated used male animals in this grant period. In the next grant period, the effects of female gender will be systematically examined.

1) Unloading causes cardiac atrophy. We first compared the effects of the duration of cardiac unloading on the magnitude of cardiac atrophy (n=12 per group): LV/body weight was 2.4 ± 0.1 vs 1.3 ± 0.1 g/kg,$p<0.05$; in control and unloaded hearts 2-wks post transplant, and 2.0 ± 0.2 vs 0.6 ± 0.2 g/kg, $p<0.01$, 5-wks post transplant. By inspection prior, all transplanted hearts were flaccid but were beating in situ (range 150-320 bpm). By histologic examinination, neither group of unloaded showed evidence of myocardial inflammation or fibrosis in comparison with controls. Thus, heterotopic transplantation can be exploited as a model of in vivo cardiac unloading of beating hearts and mild-to-severe degrees of cardiac atrophy.

2) Unloading of normal hearts promotes down-regulation of SERCA2.

Rationale: A key hypothesis of this grant is that the molecular plasticity of the heart is limited to adapt to changes in load outside normal boundaries. This hypothesis is consistent with the observations of Thomason (1992), who reported increased expression of the fetal isoform §-myosin heavy chain in spaceflown rats, and Depre et al. (1998) who confirmed enhanced expression of §-myosin heavy chain and reported a fetal pattern of increased expression of the protooncogene *c-fos* and the GLUT4 glucose transporter in rat hearts subjected to 28 days of heterotopic transplantation.

Results: In this project, left ventricular mRNA levels were measured by Northern blotting and expressed relative to levels of GAPDH following published methods (Tajima et al. 1999). The results are shown in the following table. Progressive cardiac unloading is associated with upregulation of β-myosin heavy chain. Cardiac unloading promotes a fetal pattern of reexpression of atrial natriuretic peptide as well as the progressive down-regulation of the key Ca^{2+} regulatory ATPase, SERCA2. Using Western blotting, we also confirmed that SERCA2 protein levels are also depressed. Measurements of expression of the $Na^{2+}Ca^{2+}$ exchanger ,Na^{+}-H^{+} exchanger and phospholamban will be completed soon. The findings were presented as abstract at the 1999 American College of Cardiology Scientific Sessions (Hasan et al. 1999).

Effects of Cardiac Unloading on Left Ventricular Gene Expression.

	Short Duration: 2 Wk Post Transplant N = 4 per group			Long Duration: 5 Weeks Post Transplant N = 12 per group		
	Control	Unloaded	P Value	Control	Unloaded	P Value
ANP (% control)	100+65	220+60	p=0.1	100+38	178+48	p < 0.05
β-MHC (% con)	100+52	183+67	p=0.1	100+22	218+19	p < 0.05
SERCA2 (% con)	100+21	81+17	p=NS	100 +16	44+13	p < 0.05

Mean values and standard error. Densitometric values were normalized to GAPDH. For comparison, values are expressed relative to the control values. ANP, atrial natriuretic peptide; β-MHC, β-myosin heavy chain (the slow myosin ATPase which is the fetal isofrom); SERCA2, sarcoplasmic reticulum ATPase.

2) Contractile reserve is depressed in myocytes from unloaded hearts.

Rationale: The finding of the downregulation of SERCA2 in unloaded hearts leads to the question: Is contractile function depressed in normal hearts subjected to prolonged unloading? The quantitation of intrinsic cardiac mechanical performance in normal versus unloaded hearts in the intact animal is problematic due to the marked difference in loading conditions, as well as geometric disparities. This favors the approach of study of isolated myocyte contractility. Ritter et al. (Ritter et al. 2000) studied contractility and Ca^{2+} transients in fluo-3-loaded myocytes after short duration (5 days) unloading in the heterotopic abdominal mouse transplantation model. Although cell size was smaller consistent with atrophy, a slight increase in contractility and fractional cell shortening were observed in unloaded myocytes. These findings are consistent with our molecular biology studies cited above which observed no depression of SERCA2 after short duration unloading. Ritter et al. noted that a reduction in cell volume with maintenance of cell membrane Ca^{2+} pumps is likely to contribute to an increase in the Ca^{2+} transient during short duration unloading. In this grant period, we chose to study contractile reserve in isolated left ventricular myocytes loaded with the Ca^{2+} indicator fluo-3 from long-duration transplanted hearts and control hearts, and we used the fluoresence-video microscopy techniques described above that are used in isolated mouse myocytes.

Results: Simultaneous cell shortening and calibrated intracellular Ca^{2+} transients in myocytes from unloaded hearts and controls were measured under basal conditions of field stimulation 0.5 Hz and 35oC. The table shows baseline characteristics of myocytes from control and unloaded hearts (10-12 experiments per group):

Baseline Characteristics of Isolated Myocytes: Contractility and Ca2+ Transients

	Control	Transplanted	P Values
LV/BW (mg/g)	3.1+0.3	1.3+0.2	p<0.01
Myocyte area (μm2)	3267±168	3043±162	p<0.01
Diastolic cell length(μm)	123±4	104±4	P<0.01
Fractional cell shortening (%)	5.7±0.8	5.8±0.9	NS

Time to peak shortening (ms)	132±5	141±6	NS
Time to 50% relengthening (ms)	72±4	92±9	p<0.05
Peak-systolic Ca_{2+} (nmol/L)	425±34	432±25	NS
End-diastolic Ca_{2+} (nmol/L)	72±4	77±3	NS
Time to peak Ca_{2+} (ms)	40±1	45±2	NS
Time to 50% decline in Ca_{2+} (ms)	96±5	111±5	p<0.05

To examine Ca_{2+}-dependent contractile reserve at a higher work state, we abruptly stimulated myocytes from normal and unloaded hearts with stepped increases in perfusate Ca_{2+} concentration at constant pacing frequency. The left figure shows that myocytes from transplanted hearts, compared to controls, failed to increase contraction (fractional cell shortening) in response to stepped elevation of perfusate $[Ca_{2+}]o$. The right figure shows the relationship between cell shortening and calibrated peak-systolic intracellular $[Ca_{2+}]i$ measured at each level of extracellular Ca_{2+}. The data show that the impaired contractile reserve in myocytes from unloaded hearts is related to the inability to augment intracellular systolic Ca_{2+} during this challenge. These data have been submitted as abstract to the 2000 American Heart Association Scientific Sessions.

3) Growth hormone as a potential countermeasure.

Rationale: Hormonal interventions which promote short-term enhancement of contractility, restoration of cardiac mass, or both, may be beneficial in human spaceflight when challenge by a higher gravitational environment is anticipated after long duration microgravity adjustment (e.g. return to earth or entry to Mars gravitational field). Due to the limits of human experimentation in human spaceflight missions, it is clearly desirable to consider pharmacologic interventions which are already used safely in human clinical medicine. We have previously studied short-term (2-week) therapy with recombinant human growth hormone in rodents with post-infarction cardiomyopathy, and reported that therapy with a dose that did not increase left ventricular mass was sufficient to increase SERCA2 expression and restore impaired cardiac performance in vivo and in isolated myocytes (Tajima et al. 1998). In this grant period, we completed experiments in which recombinant growth hormone, in comparison with placebo injections, was administered to animals followed heterotopic transplantation. Drug or placebo was administered for 2 weeks, during week 3-5 following transplantation (n=5 per group).

Results: Similar to our prior study in postinfarct rats, the dosing regimen was sufficient to increase circulating levels of IGF but did not promote hypertrophy in either the in situ control or unloaded hearts. Studies were performed in isolated myocytes from each group (n=10-12 experiments per group) using the protocols described above. Growth hormone therapy caused a slight increase in SERCA2 expression but did not normalize message or protein levels in unloaded hearts compared with in situ control hearts. Myocytes from unloaded hearts treated with growth hormone, in comparison with unloaded hearts treated with placebo, showed improvement in the kinetics of both time to peak shortening ($p<0.01$) and the time to 50% decline in the Ca_{2+} transient ($p<0.01$). However, this countermeasure was not sufficient to restore Ca_{2+}-dependent contractile reserve.

E. Load regulates Pol II activation, via the protein kinase, Cdk7

Rationale: Phosphorylation of RNA polymerase II is required for mRNA elongation, and is known to involve the kinase Cdk7, acting on the pol II carboxy-terminal domain. It was unknown whether pol II phosphorylation is altered by interventions that affect cardiac mass.

Results: Pol II phosphorylation in cardiac muscle was shown to be markedly increased by mechanical load, ascribable in part to the upregulation of Cdk7. In cultured myocytes, phenylephrine (an alpha-1-adrenergic agonist that suffices for hypertrophy) stimulation similarly enhanced pol II phosphorylation and Cdk7 expression. Signaling proteins that mimic (Ras-GAP) or block (N17 Ras) the phenylephrine effect increased or decreased pol II phosphorylation, in parallel with their effects on total RNA and protein synthesis. Deletion of the pol II phosphorylation domain prevented the ability of pol II to mediate the increase.

F. Load regulates the mitogen-activated protein kinase, TAK1

Rationale: All three terminal branches of the mitogen-activated protein kinase superfamily (ERK, JNK, p38) have been implicated in cardiac growth circumstantially or via cell culture studies, but nothing is known of their role in the intact animal, and the exact route through which they are activated by load is unknown.

Results: TAK1 kinase activity, which functions upstream of JNK and p38, was induced as a late response to mechanical load. Four independent transgenic mouse lines were made that mimic the chronic increase in TAK1 activity. All resulted in fulminant heart failure. The increase in TAK1 activity seen with load can by itself trigger cardiac muscle hypertrophy, fibrosis, apoptosis, gene reprogramming, and dysfunction.

G. Direct cloning of load-related genes by subtraction hybridization.

Rationale: No hypothesis-driven "candidate gene" approach can survey more than a handful of heart-expressed genes, among the known tens of thousands, for differences between two states. Suppressive subtractive hybridization is a method for selectively isolating the differentially expressed genes, with numerous advantages by comparison to earlier methods.

Results: cDNA libraries were created from neonatal versus adult myocardium, and from aortic-banded versus sham-operated hearts, by Dr. Maha Abdellatif, the collaborating investigator. Subtractive hybridization elicited more than 50 differentially expressed genes, beyond those that have been reported previously to be targets of load (Johnatty et al., 2000): genes directly involved in **transcription** (histone H2A.Z, cardiac ankryin repeat protein [CARP] Bop2, CDC10, quaking, DNA helix-destabilizing protein, and high mobility group-2 [HMG-2]); genes directly involved in **translation** (p68 RNA helicase, ribosomal proteins, L23a, L7a, S18 and L3, heterogenous ribonucleoprotein [hnRNP] A, C, F, and elongation factor 1-alpha [EF1α]); proteins that directly form (desmin, gamma actin, and lamin B1) or regulate the formation of (thymosin beta-4, pr22, Cyr61, membrane glycoprotein-type A, osteoblast specific factor [OSF-2], collagen, Mena+, tropoelastin and integrin-linked kinase [ILK]), the **cytoskeleton**; and genes involved in **signaling** (Rap1B, protein phosphatase 1γ [PP1γ], inhibitor protein phosphatase 2A [IPP2A], mss4, dynamin-like protein 1 (DLP-1) and the putative mechanosensor, ILK.

II. Implications of Project Findings for Future Research

Directionally similar changes in gene expression occur during BOTH increases and decreases in cardiac muscle cell size, indicating these are reflective of cell remodeling per se. This paradigm shift has important predictive implications for future hypothesis-testing and for specific counter-measures, as a number of adverse pathways affecting contractility, cardiac compliance, and even cell survival are activated as part of the known hypertrophic gene program.

Studies in isolated myocytes from hearts with long duration unloading show that the kinetics of relaxation and decline of the Ca^{2+} transient are slowed, consistent with the reduced expression of SERCA2. Under baseline conditions, the amplitudes of shortening and the Ca^{2+} transient are preserved. However, when the myocytes from unloaded hearts are challenged by a high work state, Ca^{2+}-dependent contractile reserve is severely depressed. Considering our short and long duration studies and Ritter's short duration studies, the data show that functional effects of unloading in normal hearts are time-dependent: the effects of long-duration unloading on cardiac gene expression and contractility differ from short-duration experiments. Thus, there is clear need for more extensive, mechanistic studies of long-duration unloading.

In the model of cardiac unloading, an initial dose regimen with recombinant growth hormone was sufficient for an encouraging biological end-point (increased SERCA2 expression) but not for preservation of cardiac mass or contractile reserve. Hence, therapy with higher levels of growth hormone merits testing. As an alternative approach, complementary agonists including α–adrenergic stimulation should be assessed, singly or in combination.

All interventions that affect cardiac mass affect pol II phosphorylation, which in turn is necessary for the increase in RNA content. Hence, stimulation of pol II phosphorylation (including by alpha-1-adrenergic agonists) is a potential means to augment or preserve cardiac mass.

Mechanical load regulates TAK1 activity. This kinase leads directly to cardiac muscle cell death. Given the delayed timecourse, TAK1 activation is presumably indirect, such as in response to a time-dependent secreted factor. Chronic changes in TAK1 activity affect cardiac mass and function. Thus, further work is needed to address the identification of secreted factors responsible for TAK1 activation, the identification and testing of TAK1 inhibitors, and the earliest steps in TAK1 activation.

Many more genes exist, that are targets of load-dependent pathways, than have been identified thus far assaying candidates manually, one at a time. High-throughput methods can contribute importantly to the molecular dissection of altered growth states.

Appendix

A. Project Research Data

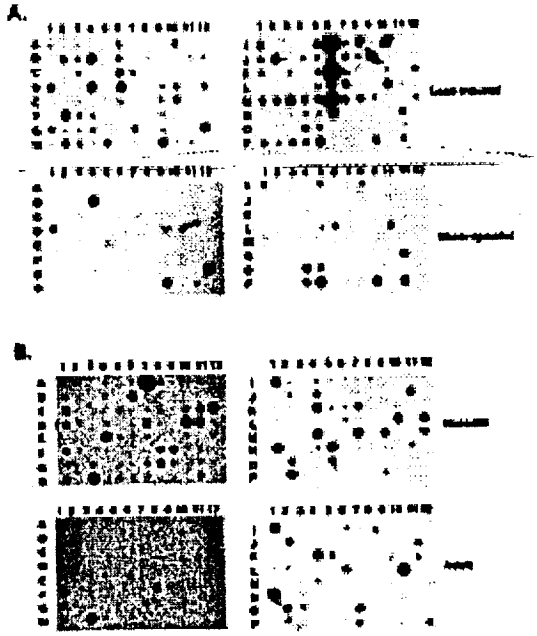


Fig 1. Direct cloning of load-regulated genes. A. Analysis of 192 clones from a hypertrophy-enriched, subtracted cDNA library, using 32P-labeled hypertrophy- enriched (top) vs control-enriched (bottom) total cDNA. B. Additonal load-regulated genes were identified by screening for genes preferentially expressed in newborn versus adult myocardium.

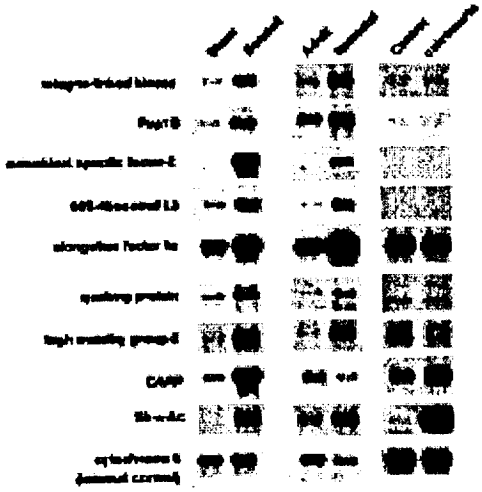


Fig 2. Northern blot analysis of 8 novel load-regulated cardiac genes, derived from the subtracted cDNA libraries. Lane 1: sham operation. Lane 2: increased load. Lane 3: adult heart. Lane 4: neonatal heart. Lane 5: control. Lane 6: calcineurin (a transgenic model with a load-independent increase in mass)

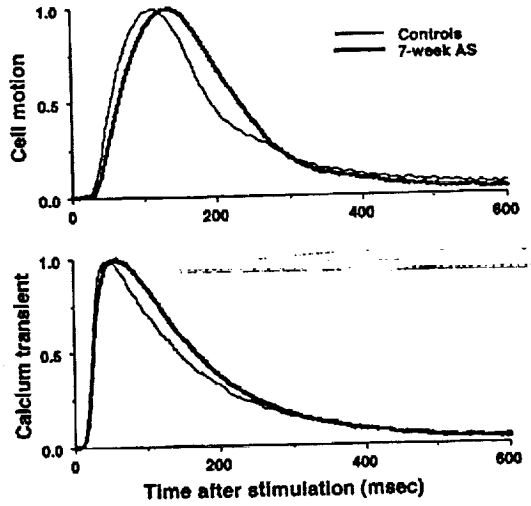


Fig 3. Alterations of cardiac myocyte contractility and intracellular calcium after altered load. Each tracing represents a signal-averaged recording of 7-week AS (n=30) and control (n=35) myocytes. Top, Myocyte contraction. Bottom, [Ca2+]i transients. Systolic myocyte shortening is shown as an upward deflection. The 7-week AS myocytes are characterized by slight depression of the velocity of contraction and relaxation and slowed decay of the [Ca2+]i transient.

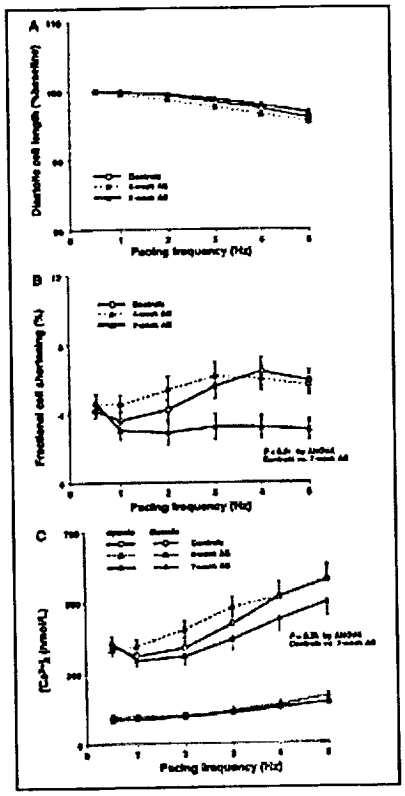


Figure 4. Frequency-dependent contractile reserve. A, Relationships between pacing frequency and diastolic cell length expressed as percent of baseline value. B, Relationships between pacing frequency and fractional cell shortening. C, Relationships between pacing frequency and peak-systolic and end-diastolic [Ca2+]i. Frequency-dependent contractile reserve is depressed in 7-week AS myocytes.

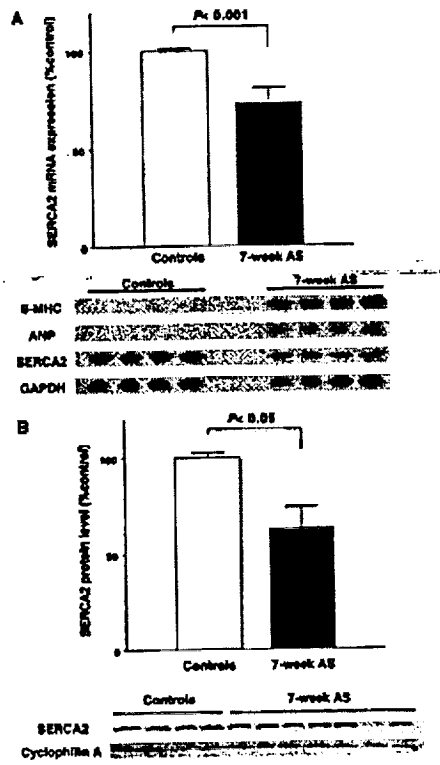


Figure 5. A, mRNA levels of SERCA2 normalized to GAPDH in LV tissue from 7-week AS and control mice and representative Northern blots of ß-MHC, ANP, SERCA2, and GAPDH. B, Levels of SERCA2 protein normalized to cyclophilin A in myocytes from 7-week AS and control mice.

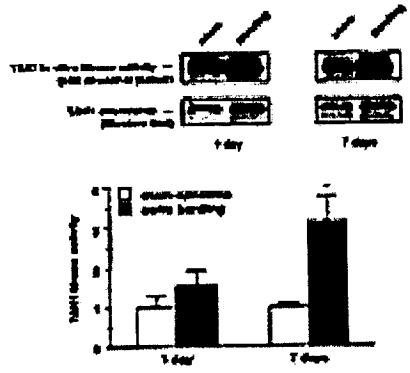


Figure 6. Activation of the protein kinase, TAK1, as at late response to altered mechanical load. Above, coupled immune complex kinase assays. TAK1 was immunoprecipitated and incubated with recombinant p38 MAP kinase as the substrate, in the presence of recombinant MKK6. Below, mean ± standard error.

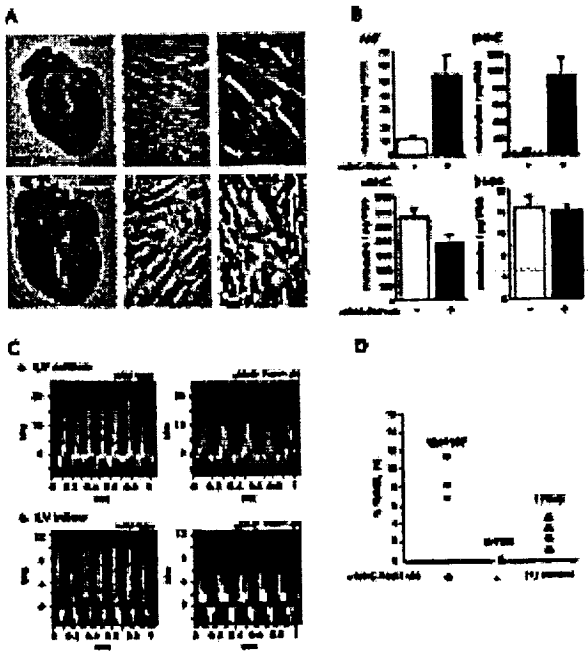


Figure 7. The load-regulated kinase, TAK1, is sufficient to impair cardiac function and induce muscle cell death. A, Hematoxylin-eosin-stained sections. B. Gene expression, by quantitative RT-PCR. C, Cardiac function, by Doppler-echocardiography. D, Programmed cell death (apoptosis); mouse mammary tissue was used as the positive control, and gave the expected prevalence of TUNEL staining.

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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

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Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated Microgravity

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EXECUTIVE SUMMARY

The Cardiovascular Alterations Team has conducted studies to determine what alterations in hemodynamic regulation result from sixteen days of simulated microgravity exposure in normal human subjects. In this project we made additional measurements on these same study subjects in order to determine whether there is an increase in susceptibility to ventricular arrhythmias resulting from simulated microgravity exposure.

Numerous anecdotal and documented reports from the past 30 years suggest that the incidence of ventricular arrhythmias among astronauts is increased during space flight. For example, documented runs of ventricular tachycardia have been recorded from crew members of Skylab and Mir [Charles JB, Bungo MW, and Fortner GW, 1994, Fritsch-Yelle, *et al.,* 1998], there was much attention given by the lay press to Mir Commander Vasily Tsibliyev's complaints of heart rhythm irregularities in July of 1997, and cardiovascular mechanisms may have been causal in the recent death of an experimental primate shortly after return from space [Richard Grindeland, Bion 11 Project Scientist, personal communication]. In 1986, a Mir cosmonaut, Alexander Laveikin, was brought home and replaced with an alternate cosmonaut as a result of cardiac dysrhythmias that began during extravehicular activity [Charles, 1998]. Furthermore, at a joint NASA/NSBRI workshop held in January 1998, cardiac arrhythmias were identified as the highest priority cardiovascular risk to a human Mars mission [Workshop to Develop Critical Path Roadmap, 1998]. Despite the evidence for the risk of a potentially lethal arrhythmia resulting from microgravity exposure, the effects of space flight and the associated physiologic stresses on cardiac conduction processes are not known, and an increase in cardiac susceptibility to arrhythmias has never been quantified.

In this study we found that 16 days of head down bed rest appears to increase the incidence of microvolt level T wave alternans, which reverts to baseline levels 2-3 days after the bed rest period. This is the first data obtained under control conditions which indicates that simulated microgravity alters cardiac electrical processes. The presence of T wave alternans (although with a lower onset heart rate than observed) in clinical patient populations has been found to indicate an increased risk of ventricular arrhythmias.

The data presented here indicate the need to further investigate the effect of space flight on the heart's susceptiblity to ventricular arrhythmias, and if necessary develop appropriate countermeasures.

Microvolt level T wave alternans testing developed under NASA and NSBRI support has now been successfully commercialized and was FDA cleared in April 1999 as a non-invasive means of identifying patients at increased risk of ventricular arrhythmias and sudden cardiac death. Three hundred thousand Americans die each year of sudden cardiac death. Effective treatment is available in the form of the implantable cardioverter/defibrillator. The problem has been that until now there has not been an effective means of identifying who is at risk. T wave alternans testing is now in clinical use and promises to have a major role in reducing sudden cardiac death.

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PROJECT RESEARCH ACTIVITY

Background

Arrhythmias During Space Flight

A variety of heart rhythm disturbances have been observed in astronauts during and after spaceflight. Occasional premature ventricular contractions were seen in Gemini and Apollo missions (4,6). Reports indicate that all crewmembers in the Skylab series demonstrated some form of rhythm disturbance (4,5) and one individual experienced a five-beat run of ventricular tachycardia. The incidence of arrhythmias was higher during flight than during pre-flight testing and higher than would be expected in a random sampling of a healthy population. Cardiac arrhythmias have also been seen during Shuttle flights (4) and on Mir. Analysis of nine 24-hour Holter monitor recordings obtained during long-term spaceflight on Mir revealed one 14 beat run of ventricular tachycardia (3). Two Mir missions have undergone major changes in crew composition and/or responsibilities due to cardiac dysrhythmias (26). Furthermore, a research primate recently died suddenly shortly after returning to earth from extended spaceflight, with cardiac dysrhythmic mechanisms suspected as a possible cause (27).

Thus, there is anecdotal evidence suggesting that spaceflight might be associated with an increased susceptibility to potentially life-threatening ventricular arrhythmias. Furthermore, ventricular arrhythmias during spaceflight may be of increasing concern in the future as older individuals are involved in spaceflight and as the duration of missions lengthen. In fact, at a joint National Aeronautics and Space Administration & National Space Biomedical Research Institute workshop in January 1998, cardiac arrhythmias were identified as one of the leading potential cardiovascular risks to a human Mars exploration mission (28). Older individuals have a greater statistical likelihood of having underlying structural heart disease, in particular coronary artery disease, and thus will be at greater risk for heart rhythm disturbances. As the duration of missions lengthen, there is an increasing likelihood that a susceptibility to ventricular arrhythmias will actually result in an arrhythmic episode. Also, longer duration spaceflights may result in a larger perturbation to cardiac electrical processes. It is therefore important to investigate quantitatively the alterations in cardiac electrical stability associated with exposure to microgravity. The potential lethal arrhythmic risk for astronauts is sustained ventricular tachycardia or ventricular fibrillation. Non-sustained ventricular tachycardia could cause syncope.

T-Wave Alternans Analysis

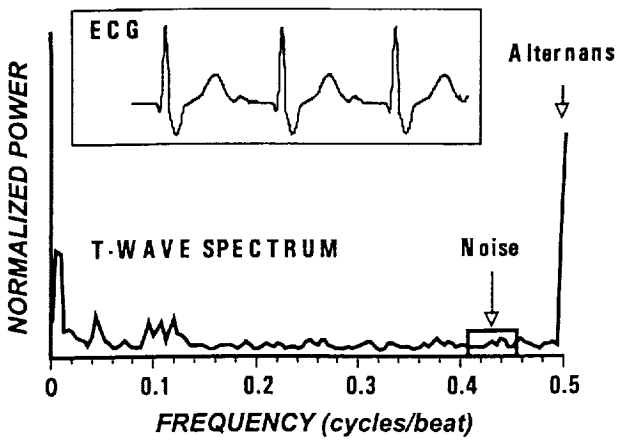


Figure 1. Representative example of the use of a computer algorithm to detect low-level alternans (From 48).

In the bed rest study reported here, we investigated whether simulated spaceflight results in a decrease in cardiac electrical stability by employing a novel methodology called T wave alternans analysis. Electrical alternans is a beat-to-beat alteration in ECG morphology which follows an A-B-A-B type pattern. Visually apparent electrical alternans is a very rare clinical finding. (Electrocardiographic alternans can be seen in the setting of pericardial effusion. In this case, the alternans is a result of the entire heart mechanically alternating its motion leading to rotation of the electrical axis of the heart and not a result of alternation of electrical conduction processes in the heart. We do not consider such electrocardiographic alternans here.) We hypothesized that very subtle electrical alternans in which the morphology of electrocardiographic complexes varied only by microvolt amounts from one beat to the next may be present in individuals at risk for ventricular arrhythmias. We developed a power spectral method (48) of detecting such low levels of electrical alternans from the analysis of 128 consecutive ECG complexes (Figure 1). The power spectrum is computed by Fourier analysis of the beat-to-beat fluctuations in the amplitude of each sample point of the 128 time aligned ECG complexes. The power spectra obtained from sample points within a given section of the ECG complex (e.g., the T wave) may be summed to obtain a measure of alternans for a portion of that complex (e.g., T wave alternans). The height of the last point in the power spectrum (corresponding to a frequency of 0.5 cycles per beat – the frequency of alternans) above the baseline level in an adjacent reference 'noise' band is the square of the alternans voltage (V_{alt}). The number of standard deviations of the 'noise' by which the height of the last sample point in the power spectrum exceeds the mean level of the baseline noise is called the alternans ratio (K). K is a measure of the statistical significance of the alternans measured. Generally, we require K to exceed three in order to determine with confidence that alternans is present.

In animal studies (49), we showed that the presence of T wave alternans correlated with enhanced susceptibility to ventricular arrhythmias as measured by the ventricular fibrillation threshold during a variety of different interventions including systemic hypothermia, coronary artery ligation, and tachycardia. These data involving 120 paired measurements show that the relationship between T wave alternans and cardiac electrical stability holds regardless of the intervention used to alter susceptibility.

Subsequently, we conducted a study in patients undergoing invasive electrophysiologic testing (EP) at the Massachusetts General Hospital (50). Invasive EP is a procedure used in patients thought to be at high risk of ventricular arrhythmias. During EP, catheter electrodes are placed in the patient's heart, and a deliberate attempt is made to induce ventricular arrhythmias in order to determine a patient's susceptibility. In this study, prior to EP, we recorded ECG signals during atrial pacing and analyzed the signals for the presence of alternans. We compared the presence of significant levels of T wave alternans with the outcome of EP and 20 month arrhythmia-free survival. The arrhythmia-free survival data are shown in Figure 2. Patients without alternans had approximately a 95% rate of arrhythmia-free survival, whereas the patients with significant levels of alternans had only a 20% rate of arrhythmia-free survival. The presence of T wave alternans was equivalent to EP as a predictor of arrhythmia-free survival. Measurement of T wave electrical alternans is a non-invasive test, whereas EP is highly invasive, expensive, and not risk free.

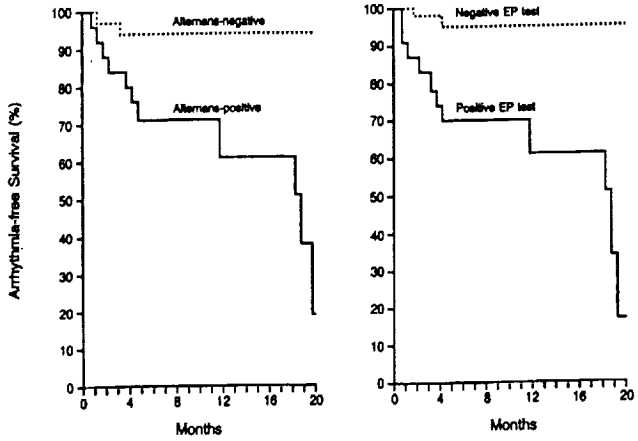


Figure 2: T wave alternans and results of electrophysiologic testing (EP) in relation to arrhythmia-free survival among 66 patients (From 50).

In order for T wave electrical alternans to be a sensitive measure of susceptibility to ventricular arrhythmias, the heart rate must be elevated. In the clinical study described above, heart rate was elevated by means of atrial pacing. In order for measurement of T wave alternans to be a fully non-invasive measure, heart rate must be elevated using non-invasive means. To accomplish this task, MIT licensed the alternans technology to a startup company, Cambridge Heart Inc., which has now successfully developed instrumentation (CH 2000) for measuring electrical alternans during bicycle or treadmill exercise (48). Although exercise introduces significant amounts of noise artifact which tend to obscure microvolt level T wave alternans, a combination of techniques involving the use of multi-contact electrodes, bicycle pedaling rate at 1/3 or 2/3 of the subject's heart rate, and noise reduction algorithms reduces the noise to a level where T wave alternans corresponding to an amplitude of one or two microvolts may be reliably measured during bicycle exercise. In patients susceptible to ventricular arrhythmias, sustained alternans (continuous alternans with $K > 3$ and $V_{alt} > 1.9$ microvolts) occurs above a subject specific heart rate threshold (48) [see Figure 3]. Heart rate thresholds under 110 beats per minute are believed to be significant indicators of risk.

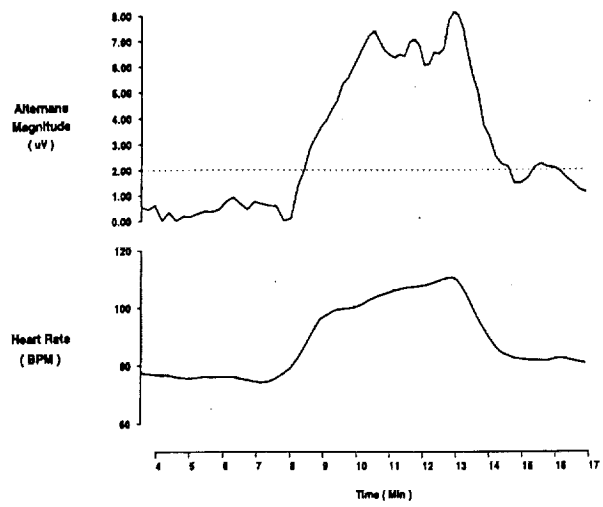


Figure 3: Plot of alternans magnitude and heart rate during the course of bicycle exercise (From 48).

Exercise induced TWA was tested as a predictor of arrhythmic events in 95 patients with implanted cardioverter defibrillators [ICDs] (51). The following arrhythmia stratification tests were performed: T wave alternans, electrophysiology testing, left ventricular ejection fraction, Holter monitoring for the presence of heart rate variability and non-sustained ventricular tachycardia, QT dispersion, and signal averaged ECG. Of these seven stratifiers TWA ($p < 0.006$) and left ventricular ejection fraction ($p < 0.04$) were the only statistically significant univariate predictors of appropriate ICD discharge as documented by review of the stored electrograms. On multivariate analysis TWA was the only statistically significant independent predictor of appropriate ICD discharge. This study shows that TWA is a powerful noninvasive predictor of tachyarrhythmic events superior to even electrophysiologic testing in this population.

In a multi-center trial of patients undergoing EP testing exercise induced TWA was compared to EP as a predictor of ventricular arrhythmic events and sudden cardiac death. Preliminary results reported from this trial (52) demonstrated that TWA (Relative Risk 11, $p < 0.001$) measured fully non-invasively was equivalent to or superior to invasive EP (Relative Risk 3.1, $p < 0.01$). On the basis of this trial the FDA cleared TWA as the only test it currently recognizes as a predictor of susceptibility to ventricular arrhythmias and sudden cardiac death.

In another study of 107 patients with congestive heart failure but with no prior history of ventricular arrhythmias, TWA identified all of the patients with subsequent arrhythmic events (53). In this study CHF was superior to all the other non-invasive risk stratifiers (presence of non-sustained VT on Holter monitoring, QT dispersion, heart rate variability, baroreceptor sensitivity, signal average ECG, and left ventricular ejection fraction).

Thus there is now substantial evidence that TWA is the most accurate non-invasive predictor of susceptibility to ventricular arrhythmias and sudden cardiac death in a variety of patient populations.

Experimental Results

Experimental Protocol. Following the screening procedures, subjects were admitted to the Brigham and Women's Hospital General Clinical Research Center. They spent three (subjects 1-4) or five (subjects 5-15) days undergoing baseline testing and equilibrating to an isocaloric diet consisting of 200 mEq sodium, 100 mEq potassium, and 2500 ml fluid.

Subjects then underwent a tilt-stand protocol. Following the tilt-stand test measurements for TWA were made in the same subjects while they rode on a stationary exercise bicycle and pedaled at a rate (in revolutions per minute) that was 1/3 of their heart rate throughout the exercise period for noise reduction purposes. The exercise stress test was conducted at up to 70% of the subjects predicted maximum heart rate (maximum predicted heart rate is defined as 220 bpm minus the subject's age in years). Measurements were made using a CH 2000 (Cambridge Heart Inc., Bedford, MA) system. ECG recordings were made using multi-contact electrodes placed at locations which enabled recording both vector and precordial electrocardiograms. TWA was measured using the Spectral Method. The resulting trend plots were reviewed in a blinded fashion to determine whether sustained alternans occurred during the period of the test and, if present, to determine the onset heart rate. Sustained alternans is defined as TWA which is consistently present above a subject-specific onset heart rate. The TWA alternans measurement was made again 16 days of head-down tilt bed rest following a second tilt-stand test, and again two to three days after the completion of bed rest.

Of the fifteen subjects who participated in this study, 11 underwent successful testing for TWA. Four of the 11 subjects (5, 7, 10, 11) who were tested for TWA received the alpha-1 agonist drug midodrine (Roberts Pharmaceutical, Inc.) on the final day of bed rest about four hours prior to testing as part of a randomized double blind trial of this drug as a countermeasure against orthostatic intolerance. As the half-life of this drug is four hours, and the dose given was relatively small, it was felt this would have a minimal effect on the TWA results.

Results. Data from the eleven subjects who completed TWA testing was analyzed in a blinded fashion. The results of the TWA tests on the 11 subjects are summarized in the Table. Three subjects did not have sustained TWA prior to bed rest developed sustained alternans immediately after bed rest. In these three subjects sustained TWA disappeared two or three days after bed rest. In one subject sustained TWA was present prior to bed rest, disappeared immediately after bed rest, and reappeared three days after bed rest.

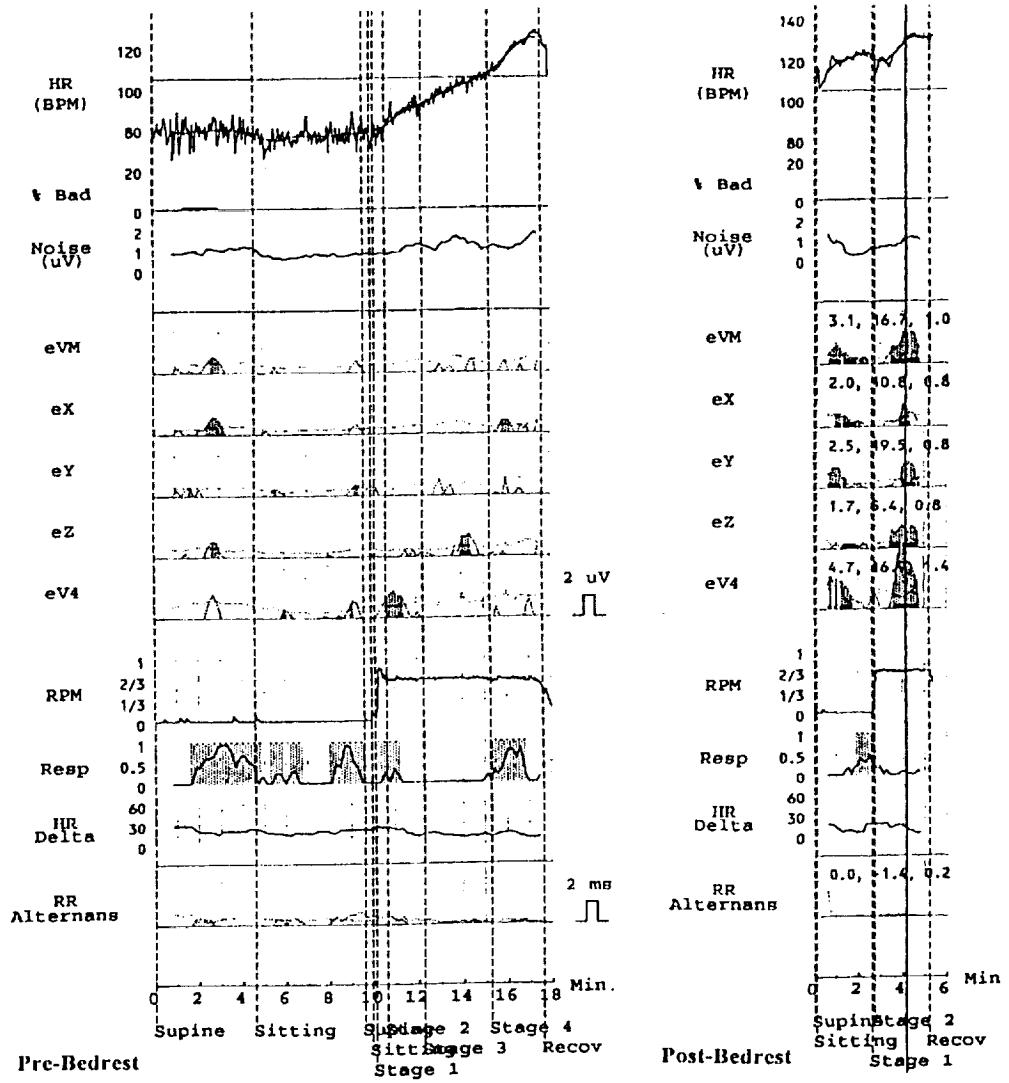
Table. Results of TWA tests in eleven subjects before, within three hours after and two or three days after head-down tilt bed rest. TWA70+ = Positive using a threshold of 70% of maximum predicted heart rate. All studies were read in a blinded fashion. Subjects 5, 7, 10, and 11 received Midodrine 5 mg orally four hours prior to TWA testing.

Subject	Pre-Bed Rest Control (Ambulatory)	Immediately Post-Bed Rest	Post-Bed Rest Control (Ambulatory)
1	NEG	NEG *(Recovery TWA)*	NEG
2	NEG	Onset 130 TWA70+	NEG
3	NEG	Onset 120 TWA70+	NEG
4	NEG	NEG	NEG
5	Onset 120 TWA70+	NEG	Onset 125 TWA70+
6	NEG	NEG	NEG *(NSA* at Peak HR)*
7	NEG	NEG *(NSA* at Peak HR)*	NEG
8	NEG	Onset 120 TWA70+	NEG
9	NEG	NEG *(NSA* at Peak HR)*	Onset 125 TWA70+
10	NEG	NEG	NEG
11	Not Done	**NEG**	NEG *(Recovery TWA)*

*NSA = Non-Sustained Alternans.

Note that none of the onset heart rates for sustained TWA was less than or equal to the positive heart rate threshold (PosHRT) of 110 beats per minute (below which sustained alternans is usually regarded to be clinically significant). However using the alternative definition of the PosHRT = 70% of maximum predicted heart rate, then 3/11 subjects who were TWA70- pre-bed rest became TWA70+ post-bed rest and reverted to TWA70- pre-discharge. Five of 11 subjects exhibited Non-Sustained Alternans (NSA) or Recovery TWA either immediately post-bed rest or two or three days later, while no subjects exhibited these findings pre-bed rest. One subject who

Figure 4 TWA traces before and immediately after bed rest in the same magnitude lead, vectors leads X, Y, Z and lead V4. In the left hand trace there i sustained alternans. In the right hand trace sustained alternans [TWA at least duration, with Valt greater than or equal to 1.9 microvolts, and K greater (indicated grey shading), consistently present above a patient specific onset present in the vector magnitude lead, lead Z and lead V4 with onset heart



was TWA70+ pre-bed rest and pre-discharge was TWA70- post-bed rest.

None of the subjects who developed TWA after bed-rest received midodrine. The one subject who had sustained TWA prior to bed-rest did receive a 5 mg dose of midodrine prior to being tested immediately following bed-rest. We cannot exclude the possibility that midodrine contributed to the elimination of sustained TWA in this individual.

Figure 4 illustrates a pre-bed rest TWA trace on the left versus a post-bed rest TWA trace in the same subject on the right. Sustained alternans was present above a patient specific heart rate post-bed rest, but was absent pre bed rest. See figure caption for details. After three days of ambulatory activity this subject had no T wave alternans.

The data from this study demonstrate that 14 or 16 days of head-down tilt bed rest may lead to the development of sustained TWA in normal subjects, although the onset heart rate is above the 110 bpm cutoff which is normally considered clinically significant. This finding provides the first evidence that simulated spaceflight has a systematic and measurable effect on myocardial electrical conduction, which may indicate an increased susceptibility to life threatening ventricular arrhythmias. Three subjects who did not have evidence of sustained TWA prior to bed rest developed TWA following bed rest. When tested two or three days later, TWA had disappeared in these subjects. From these data we have concluded that bed rest induces sustained T wave alternans in normal subjects. This induction of TWA is reversible. In addition, in one subject who had sustained TWA prior to bed rest, TWA disappeared post bed rest, and reappeared three days later. This study demonstrates that bed rest alters the myocardial repolarization processes as measured by TWA, although by the standard criteria, no subject would have been considered clinically at increased risk for sudden death.

IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

Spaceflight

Whether space flight increases susceptibility to serious heart rhythm disturbances has been identified as one of the primary cardiovascular risks. If long term space flight does predispose astronauts to potentially lethal heart rhythm disturbances this could be the pre-eminent critical astronaut safety issue. While anecdotal data suggest that space flight does increase the incidence of potentially serious heart rhythm disturbances, we have not had data collected under controlled conditions that would enable us to answer this question. The present data suggest that 16 day head down tilt bed rest alters cardiac repolarization processes so as to increase the incidence of microvolt T wave alternans. The development of T wave alternans (and with a lower onset heart rate than observed here) in other populations has been a very reliable marker of increased cardiovascular risk. The data from the present study show for the first time that simulated microgravity alters cardiac electrical processes. The present data were collected in male volunteers who primarily were young adults. In future ground based studies we will examine the incidence of T wave alternans in premenopausal women undergoing bed rest as well as in older men. We expect that older men will be the most susceptible to the development of microvolt level T wave alternans. We plan to test in this group the aldosterone blocker, spironolactone, which in heart failure populations has been shown to reduce the risk of arrhythmic death. The

hypothesis here is that both microgravity and heart failure are hyper-aldosterone states and that aldosterone through its toxic effect on the myocardium and its effects on electrolytes increases susceptibilty to ventricular arrhythmias. Thus aldosterone blockers such as spironolactone might serve as an effective countermeasure. We have also proposed testing astronauts before and after space flight to determine directly whether space flight increases the incidence of microvolt level T wave alternans.

Earth Benefits

This project, and preceding support by NASA, of the development of microvolt level T wave alternans has led to the development of microvolt level T wave alternans as a clinical tool for the identification of individuals at increased risk of ventricular arrhythmias. This technology has been successfully commercialized. In April 1999, the FDA cleared T wave alternans recognizing it as a predictor of risk for sudden cardiac death and ventricular arrhythmias, and it is now in increasing clinical use for this purpose. Sudden cardiac death claims the lives of approximately 300,000 Americans each year; one in seven Americans will ultimately die of sudden cardiac death. An effective technology to prevent sudden cardiac death is available – the implantable cardioverter defibrillator. Until now however there has not been an effective non-invasive means of identifying who is at risk. The development of microvolt level T wave alternans testing from the space research program promises to provide this means and thus lead to a dramatic reduction in mortality from sudden cardiac death.

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APPENDIX A – Project Research Data

Contained in this report and in publications listed in Appendix B

APPENDIX B – Publications

Armoundas A, Rosenbaum DS, Ruskin JN, Garan H, Cohen RJ. Prognostic significance of electrical alternans versus signal averaged electrocardiography in predicting the outcome of electrophysiological testing and arrhythmia-free survival. Heart 1998: 80: 251-256.

Armoundas AA, Osaka M, Mela T, Rosenbaum DS, Ruskin JN, Garan H, Cohen RJ. T-Wave Alternans and Dispersion of the QT Interval as Risk Stratification Markers in Patients Susceptible to Sustained Ventricular Arrhythmias. American Journal of Cardiology 1998; 82: 1127 – 1129.

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Armoundas AA, Toshihiko N, Cohen RJ. T-Wave Alternans Preceding Torsade de Pointes Ventricular Tachycardia. Circulation 2000; 101: 2550.

APPENDIX C – Publications Enclosed

Armoundas A, Rosenbaum DS, Ruskin JN, Garan H, Cohen RJ. Prognostic significance of electrical alternans versus signal averaged electrocardiography in predicting the outcome of electrophysiological testing and arrhythmia-free survival. Heart 1998: 80: 251-256.

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NSBRI RESEARCH PROGRAM
HUMAN PERFORMANCE FACTORS

Team Leader:	**Czeisler, C. A.**		**Harvard**
Czeisler, C. A.	**PI**	**Harvard**	**Circadian Entrainment, Sleep-Wake Regulation and Neurobehavioral Performance During Extended Duration Space Flight**
Kronauer, R. E.	CO-I	Harvard	
Ronda, J. M.	CO-I	Harvard	
Wright, K. P.	CO-I	Harvard	
Dinges, D. F.	**PI**	**Penn**	**Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight**
Maislin, G.	CO-I	Penn	
Mullington, J. M.	CO-I	Harvard	
Rogers, N.	CO-I	Penn	
Szuba, M. P.	CO-I	Penn	
Van Dongen, H. P.	CO-I	Penn	
Dijk, D.	**PI**	**Surrey**	**Quantitative EEG Monitoring of Vigilance: Effects of Sleep Deprivation, Circadian Phase and Sympathetic Activation**
Cajochen, C.	CO-I	Harvard	
Lockley, S. W.	CO-I	Surrey	
Brown, E.	**PI**	**Harvard**	**On-Line Analysis of Physiologic and Neurobehavioral Variables During Long-Duration Space Missions**
Jewett, M. E.	CO-I	Harvard	
Kronauer, R. E.	CO-I	Harvard	
Luithardt, H. H.	CO-I	Harvard	
Young, L. R.	**PI**	**MIT**	**Ground-Based Study and Evaluation of Principal Investigator-in-a-Box**
Szolovitz, P.	CO-I	MIT	

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

OVERALL ANNUAL REPORT FOR YEAR 3: SEPTEMBER 2000

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VII. **Synergy Project 2:** "Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and the Renin-Angiotensin System"
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PROGRAM EXECUTIVE SUMMARY

Errors in human performance cause most accidents in technology-rich environments such as commercial aviation, where two-thirds of accidents are attributable to performance errors by cockpit crews. In space, the contribution of human performance factors to mission success is even greater, since a number of fundamental aspects of the space environment compromise physiologic systems critically involved in human performance. Human factors is a broad area that includes biological limits on performance [e.g., circadian rhythms, sleep need, microgravity, radiation, environmental factors (temperature)], operational demands on performance (e.g., skilled task demands, monitoring complex automation, mission requirements), psychosocial effects on performance (e.g., effects of isolation, crew selection, family contact, crew communication/coordination) and human ergonomics (e.g., habitability, equipment design, workload, training). Initially, the Human Performance Factors, Sleep and Chronobiology Team is focused on the biological limits of human performance, particularly those compromised by specific aspects of the space environment, including microgravity, an absence of geophysical 24-h cycles, limited sleep/rest opportunities, a high level of automation and a remote, inaccessible location. Such conditions will likely be ubiquitous among the astronauts and are known to affect physiologic, behavioral and cognitive processes critically involved in human performance. These aspects of the space environment result in or require: (a) disrupted circadian entrainment; (b) dyssomnia; (c) cumulative sleep loss; (d) execution of life science research remote from the Principal Investigator (PI).

The overall strategy of the Human Performance Factors, Sleep and Chronobiology Team was based on the recognition that optimizing human performance in space can best be achieved by: (1) understanding the basic mechanisms underlying the deterioration of human neurobehavioral function in space related to these factors; and (2) developing effective countermeasures based on those mechanisms to minimize human error and optimize human performance in the highly automated space environment. Currently, for example, astronauts' sleep duration, which is one of the most fundamental determinants of their waking neurobehavioral performance, averages only 6 hours per night, and may be as low as 3 to 4 hours per night. Ground-based studies indicate that within 2 weeks, the effects of such cumulative sleep deprivation are equivalent to the effects of 48-60 hours of total sleep deprivation. Recent work elsewhere indicates that as little as 24 hours of total sleep deprivation has been reported to degrade aspects of neurobehavioral performance to a level comparable to a blood alcohol level of 0.10 percent. To counteract their difficulty sleeping, astronauts and the flight surgeons responsible for their medical care currently rely during space flight on *ad lib* self-administration of hypnotic medications that were developed for the treatment of insomnia, with 50% of crew members in dual shift operations resorting to sleeping pill use during the missions.

This integrated research team has investigated a series of novel approaches to address such human performance factors, including: the mechanisms of circadian entrainment and sleep regulation; statistical algorithms for on-line analysis of physiologic variables monitored during long-duration space missions; and the development of expert systems for the remote execution of life science experiments. These approaches were integrated with the aim of developing countermeasures and testing their efficacy. The multi-disciplinary approach adopted for study of the affected physiologic, behavioral and cognitive processes in humans (i.e., including circadian entrainment, sleep homeostasis, and decision-making processes) incorporated five team projects and two inter-team synergy projects.

Integration was achieved by thematic organization around the defining characteristics of the space environment that influence human performance. The research program of the Human Performance Factors, Sleep and Chronobiology Team was a goal-directed research program that provided an integrated contribution to the overall NSBRI mission and addressed the Institute's Aims and Objectives by: (1) Designing, implementing, and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight; (2) Defining the whole-organism integrated-physiological and neurobehavioral responses that ultimately determine these impediments and designing novel countermeasures based on these responses; (3) Establishing support technologies to maximize human performance in space and to reduce the probability of human performance failure; (4) Transferring and disseminating the advances in knowledge and technology acquired to populations on

earth in which performance is jeopardized; (5) Providing training of new scientists in the space life sciences by recruiting young scientists for active participation in the proposed ground-based research program and by mentoring these young scientists for the duration of these projects.

The research that the team has conducted is relevant for the round-the-clock work schedules (day, evening and night work) on the International Space Station, the altered sleep/wake schedule required on a Mars surface station, or any other situation in which the work-rest schedule is shifted or sleep loss is incurred. It also has relevance for ground personnel monitoring orbiting crew members who must do so around-the-clock. Through the efforts of this Program, the Human Performance, Sleep and Chronobiology Team has worked towards developing effective countermeasures to minimize human error and optimize human performance in the highly automated space environment. The research program of the Human Performance Factors, Sleep and Chronobiology Team has been a goal-directed research program that has provided an integrated contribution to the overall NSBRI mission. The results of this team effort could have an important effect on the health, safety and productivity of astronauts during extended duration missions, such as those planned for the International Space Station and for the manned mission to Mars.

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I. PROGRAM RESEARCH ACCOMPLISHMENTS

A. OVERALL PROGRAM AIMS & OBJECTIVES

Human space flight depends on the ability of crew members to maintain high-level cognitive performance and vigilance while operating and monitoring sophisticated and highly automated instrumentation in the presence of substantial physiological and psychological challenges. Despite the skill level and motivation of the astronauts, a higher frequency of human errors occurs in orbit than during training. The theme of the Human Performance Factors, Sleep and Chronobiology Team has been to elucidate the basic mechanisms responsible for the deterioration of human neurobehavioral function in space and to develop effective countermeasures to minimize human error and optimize human performance in the highly automated space environment. The team has focused on these areas:

- circadian entrainment in a controlled photic environment, including studies of intermittent bright light exposure, melatonin, and phase relations among circadian variables in subjects scheduled to an earth day (i.e., 24-h sleep-wake schedule) or Martian day (i.e., 24.6-h sleep-wake schedule (Project 1);
- sleep scheduling countermeasures to cumulative neurobehavioral deficits and growth hormone deficiency due to chronic partial sleep deprivation (Project 2);
- on-line monitoring of electroencephalographic and ocular indices of decrements in physiological alertness and performance associated with sleep loss and a supine posture (Project 3);
- statistical algorithms for on-line dynamic analysis of physiological data (Project 4).
- expert systems for remote, on-board mentoring of crew decision-making (Project 5);

B. BRIEF DESCRIPTION OF PROJECTS WITHIN PROGRAM

PROJECT 1. Circadian Entrainment, Sleep-Wake Regulation and Neurobehavioral Performance During Extended Duration Space Flight. Drs. Czeisler (Lead), HMS; Wright, HMS; Kronauer, HMS; Khalsa, HMS; and Mr. Ronda, HMS.

Sustaining high levels of performance throughout extended duration space missions requires: 1) circadian entrainment of the intrinsic longer-than-24-hour period of the human circadian pacemaker to the 24-hour day; and 2) maintenance of an appropriate phase relation of the human circadian pacemaker to the 24-h sleep-wake schedule. In space, astronauts are often exposed to light-dark cycles that are characterized by either an abnormal period, i.e., 1.5 hours during earth orbit, or by a reduced light intensity, i.e., between 10-50 lux in the angle of gaze, when the space craft is illuminated artificially and under power constraints. However, there is a critical deficiency in knowledge regarding the minimum intensity of light required to maintain the appropriate phase-relation between the 24-h sleep-wake cycle and the circadian system and thereby avoid misalignment of circadian phase. It is essential to correct this knowledge gap, since such circadian phase misalignment can result in sleep disturbances, reduced attention, gastrointestinal disorders and impaired daytime alertness.

Based on these preliminary results, three testable hypotheses were evaluated, which are critical for the development of effective countermeasures to prevent circadian misalignment: 1) that synchronization of the human circadian pacemaker will be disturbed in men and women by the reduction in LD cycle strength during space flight; 2) that synchronization of the human circadian pacemaker to the 24.6 hr day of Mars will be disturbed in men and women; 3) that this disturbed circadian synchronization will result in the secretion of the sleep-promoting hormone melatonin during the waking day, disturbed sleep, reduced growth hormone secretion, and impaired performance and daytime alertness.

To test the hypotheses, a long-term (55-day) clinical trial was carried out utilizing methodologies that allowed direct measurement of endogenous circadian phase and amplitude before, during and after extended duration exposure to an environment with a reduction in the strength of external synchronizing cues [i.e., low light

intensities].

This work has important implications for the treatment of circadian rhythm sleep disorders, such as delayed sleep phase syndrome and shift-work dyssomnia, which are anticipated to have a high incidence and prevalence during extended duration space flight. It is anticipated that the results of the investigators' NASA/NIA-sponsored clinical trial of melatonin as a potential sleep-promoting countermeasure will be integrated with the results from these studies into a predictive mathematical model. The results of the proposed research could have a profound effect on the health, productivity and safety of astronauts during extended missions, such as those planned for the International Space Station and for the manned mission to Mars.

PROJECT 2. Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight. Drs. Dinges, UPENN; Maislin, UPENN; Van Dongen, UPENN; Rogers, UPENN; Szuba, UPENN; Mullington, HMS.

The performance capability of astronauts during extended-duration space flight depends heavily on achieving daily recovery through adequate sleep. Studies in astronauts using daily logs, wrist actigraphs and ambulatory polysomnography provide convergent evidence that astronaut sleep is restricted in space flight to averages between 4 hr per day and 6.5 hr per day (mean = 6.0 hr per day). There appear to be multiple causes for the restriction, including endogenous disturbances of sleep (microgravity, motion sickness, stress, circadian rhythms), environmental disruptions of sleep (noise, temperature, light), and curtailment of sleep due to the extended work demands that often accompany space flight operations. An average of 6 hr sleep per day poses a risk to astronauts. Ground-based experiments in multiple laboratories have demonstrated that daily nocturnal sleep durations in the range of 4 – 6.5 hours result in progressive fatigue, as well as neurobehavioral performance deficits that include lapses of attention, slowed response times, reduced cognitive throughput, degradation of complex problem solving, impaired learning, and alterations of physiologic functions. Modest rates of impairment after 1-3 days of sleep restriction evolve into serious deficits by 7-10 days. The mechanism through which these deficits occur is the development of cumulative homeostatic pressure for sleep across consecutive days of sleep restriction (referred to as "sleep debt"). As sleepiness increases neurobiologically, waking neurocognitive functions become unreliable. Research has shown that these cumulative performance deficits are often not accompanied by full subjective awareness of the severity of the impairment, which may explain why astronauts may not report problems when sleep is restricted. Alterations of sleep- and circadian-mediated hormonal profiles and metabolic changes have also been observed in subjects exposed to chronic sleep restriction.

To counter cumulative waking deficits from sustained sleep restriction, either the sleep drive must be met through increased duration of the major (anchor) sleep episode, and/or through the strategic use of a single daily pre-planned (preemptive) nap, which also segments one long waking episode into two shorter durations. However, there is a critical deficiency in knowledge of the effects of varying combinations of anchor sleep and nap durations that will yield the most efficient return of performance per unit time invested in sleep. The primary aim of this project was to meet this critical deficiency through use of a *response surface experimental paradigm*, testing in a dose-response manner 18 different combinations of anchor sleep and nap sleep durations for the purpose of establishing how to most effectively limit the cumulative effects of chronic sleep restriction in space operations. A search algorithm involving a two-stage regression analysis was used to test the hypothesis that the addition of a preemptive midday nap to restricted nocturnal (anchor) sleep each day will markedly attenuate the *slope* (growth) of the cumulative performance deficits developing across days. This approach was also used to test the hypothesis that the addition of a nap to anchor sleep will yield normal secretion of sleep- and circadian-mediated hormones (melatonin, cortisol, growth hormone). The experiment is the first ground-based study to utilize the slopes of cumulative neurobehavioral deficits and physiological changes across days of chronic sleep restriction, to determine the extent to which the duration of sleep per 24 hours (in the range commonly experienced by astronauts in flight) and the use of combined anchor + nap sleep opportunities each day, can prevent or attenuate the development of cumulative

fatigue and performance deficits. The response surface experimental paradigm affords a high return of information regarding the optimal way to utilize sleep in operations that inherently limit time for sleep in the space flight environment. The results of the research will contribute to the optimization of performance, productivity, safety and health during extended missions, by providing astronauts with the most efficient sleep-wake schedules. The results of this project also have important implications for optimizing work-rest scheduling in Earth-based safety-sensitive industries that must operate around the clock (e.g., transportation, military, public safety).

PROJECT 3: Quantitative EEG Monitoring of Vigilance: Effect of Sleep Deprivation, Circadian Phase, Posture and Sympathetic Activation. Drs. Dijk, HMS and University of Surrey; Cajochen, HMS.

Shuttle astronauts typically sleep only 6 to 6.5 hours per day while in orbit. This sleep loss is related to recurrent sleep cycle shifting--due to mission-dependent orbital mechanics and mission duration requirements--and associated circadian displacement of sleep, the operational demands of space flight, noise and space motion sickness. Such sleep schedules are known to produce poor subjective sleep quality, daytime sleepiness, reduced attention, negative mood, slower reaction times, and impaired daytime alertness. Countermeasures to allow crew members to obtain an adequate amount of sleep and maintain adequate neurobehavioral performance are being developed and investigated. However, it is necessary to develop methods that allow accurate and attainable in-flight monitoring of vigilance to evaluate the effectiveness of these countermeasures and to detect and predict online critical decrements in alertness/performance. There is growing evidence to indicate that sleep loss and associated decrements in neurobehavioral function are reflected in the spectral composition of the electroencephalogram (EEG) during wakefulness as well as in the incidence of slow eye movements recorded by the electro-oculogram (EOG). Furthermore, our preliminary data indicated that these changes in the EEG during wakefulness are more pronounced when subjects are in a supine posture, which mimics some of the physiologic effects of micro-gravity. Therefore, we evaluated the following hypotheses: (1) that during a 40-h period of wakefulness (i.e., one night of total sleep deprivation) neurobehavioral function deteriorates, the incidence of slow eye-movements and EEG power density in the theta frequencies increases especially in frontal areas of the brain; (2) that the sleep deprivation induced deterioration of neurobehavioral function and changes in the incidence of slow eye movements and the spectral composition of the EEG are more pronounced when subjects are in a supine position; and (3) that based on assessment of slow-eye movements and quantitative on-line topographical analyses of EEG during wakefulness an EEG and or EOG parameter can be derived/constructed that accurately predicts changes in neurobehavioral function.

If shown to be accurate, this EEG/EOG-based online monitoring of alertness/performance would serve as a practical tool to predict and prevent critical decrements in performance and alertness. It would complement investigations of the effectiveness of countermeasures based on neurobehavioral function. Understanding the relationship between EEG/EOG and neurobehavioral function could thus lead to the development of an effective system for preventing fatigue-related errors and accidents during space flight.

PROJECT 4. On-Line Analysis of Physiologic and Neurobehavioral Variables During Long Duration Space Missions. Drs. Brown, HMS; Kronauer, HMS; Jewett, HMS; Luithardt, HMS.

The goal of this project is to develop reliable statistical algorithms for on-line analysis of physiologic variables monitored during long-duration space missions. The methods will be important for detecting promptly alternations in normal physiology, administering countermeasures and for assessing their effects. Maintenance of circadian homeostasis is essential for ensuring optimal crew performance. Therefore, the project will test the hypothesis that alternations in circadian entrainment can be detected in near real-time by on-line analysis of core-temperature, melatonin, and neurobehavioral data using statistical methods designed to assess the dynamical properties of the human circadian pacemaker. The analysis will provide a continually updated profile of each circadian variable including estimates of rhythm period, amplitude, position with respect to the limit cycle. It will also characterize changes in phase relationships among circadian variables. The methods are being developed using measurements of core-temperature, melatonin and neurobehavioral data collected in Project 1. It is hypothesized that

the paradigm can be adapted to monitor non-circadian physiologic variables and that it can be incorporated easily into an expert-system (Project 5).

PROJECT 5. Artificial Intelligence Decision Aiding for Astronaut Performance of Experiments. Drs. Young, MIT; Natapoff, MIT; and Szolovits, MIT.

Space life science research is arguably the most demanding of all astronaut scientific endeavors. The already significant problems of adequate training and oversight of experiments conducted on-orbit will only increase in the era of the International Space Station, which includes increased facilities and protocols, with limited crew specialists. This project is testing the expert system's ability to meet the decision aiding needs of astronauts conducting life science experiments. By encapsulating the "heuristic reasoning" of a real principal investigator into "P.I. in a Box", an "expert system" in an on-board computer, a computer decision aid can be provided. This project tested three hypotheses, as follows. When compared to conventional crew training alone, use of an expert system will improve average time to: (1) detect the deterioration of signal quality; (2) correctly identify the source of artifactual data in a complex situation; and (3) complete a normal calibration and run of a physiological experiment. The experimental protocol includes training individuals in the Neurolab sleep experiment (related to Project 1) with randomly degraded electrophysiological signals to test whether computer aiding improves training and subsequent performance. The expert system rules build on the existing [PI] programs for troubleshooting and error detection developed for the Neurolab Sleep experiment. The expert system should result in better quality results, fewer errors and/or lost data, and more efficient use of astronaut time for space experiments.

SYNERGY PROJECT 1. Immunomodulatory Consequences of Sustained Partial Sleep Deprivation: *In vitro* and *In vivo* Indices. Drs. Mullington, HMS; Dinges, UPENN; Butel, BCM; Schwartz, BCM; Ling BCM.

In long term space flight, the issue of immune suppression and possible reactivation of latent viruses are risks that cannot be prevented by pre-flight quarantine. The possibility of reactivation of latent viruses is a serious potential hazard for the success of long-term space missions where confined conditions increase the possibility of transmitting disease. It is also known that muscle fiber degeneration occurs during prolonged space travel. Both of these processes may be exacerbated by the neuroendocrine consequences of chronic partial sleep deprivation. This project seeks to investigate the neuroendocrine and neuroimmune modulation of sustained partial sleep deprivation and furthermore to test the influence of cumulative sleep loss on the reactivation of latent viruses, and the delayed hypersensitivity response to cutaneously applied antigen testing by synergizing the efforts of three NSBRI research teams. The results of this research will integrate the teams and give them the opportunity to examine data using technologies and methodologies that come from separate and distinct scientific methodologies, and bring them together in a common protocol with a view to enhancing the potential of each team to take back information that will help in the development and implementation of countermeasure strategies and future scientific developmental plans.

SYNERGY PROJECT 2: Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and the Renin-Angiotensin System. Drs. Dijk, HMS; Williams, HMS; and Cajochen, HMS.

The main aim of this enhancement grant was to exploit the similarities in research protocols between the Human Performance Team and the Cardiovascular Alterations Team by including the assessment of outcome variables relevant to the Renal-Cardiovascular project in the research protocol of Project 3 of the Human Performance Team and by including the assessment of outcome variables relevant to the Quantitative EEG and Sleep Deprivation Project in the research protocols of Project 3 of the Cardiovascular Alterations Team. In particular, the project was designed to investigate the following two specific aims: (1) test the hypothesis that chronic partial sleep deprivation during a 17-day head down tilt bedrest experiment results in deterioration of neurobehavioral function during waking and increases in EEG power density in the theta frequencies, especially in frontal areas of the brain, as well as the nonREM-REM sleep cycle dependent modulation of heart-rate variability; and (2) test the hypothesis that acute total

sleep deprivation modifies the circadian rhythm of the renin-angiotensin system, changes the acute responsiveness of this system to posture beyond what a microgravity environment alone does and affects the nonREM-REM sleep cycle dependent modulation of heart-rate variability.

The data obtained on the waking EEG and neurobehavioral function in the chronic partial sleep deprivation experiment complement the data obtained on the effects of total sleep deprivation collected in Project 3 of the Human Performance Team. The data obtained on the renin-angiotensin levels in the acute total sleep deprivation experiment complement data obtained on the effects of chronic partial sleep deprivation collected in Project 3 of the Cardiovascular Alterations Team. The application of identical research tools and outcome measures in research protocols across the Cardiovascular and Human Performance Teams will greatly synergistically enhance the overall science return of these projects.

C. GENERAL PROGRAM STRATEGY

Given the prevalence of sleep and circadian disturbance on space missions, we have pooled our expertise and experience to identify 5 aspects of the space environment as critical to human performance in this Team Proposal. These include: (1) absence of geophysical 24-h cycles; (2) microgravity; (3) limited sleep/rest opportunities; (4) high level automation; (5) remote/inaccessible location. These aspects of the space environment result in or require: (a) disrupted circadian entrainment; (b) dyssomnia; (c) cumulative sleep loss; (d) execution of life science research remote from the Principal Investigator (PI). Although the contributions of these aspects of the space environment to human performance have been recognized, **critical deficiencies in the presently existing information** have been identified in the following areas.

Absence of geophysical 24-h cycles: The minimum light intensity needed to maintain normal circadian entrainment is not known. Predictive mathematical models integrating the effects of light exposure, circadian phase and amplitude, and sleep consolidation on cognitive performance are unavailable (Projects 1 and 2). Time series analysis methods to verify circadian entrainment on-line are not available (Project 4).

Dyssomnia of space flight: The minimum sleep duration to maintain optimal performance and optimal timing of naps to counteract sleep loss-related performance decrements is currently not known (Projects 1 and 2). Reliable electrophysiologic indicators of the performance and alertness decrements associated with sleep loss have not been identified and or validated (Project 3). Furthermore, the quantitative effect of the dyssomnia of space flight on hypothalamic-pituitary functions, such as the secretion of human growth hormone, is not known. Nor is it known how to most effectively schedule sleep to counter cumulative sleepiness and augment human growth hormone release (Project 2).

Continuous vigilance and chronic supervision of highly automated control tasks: It is currently not known how astronauts will maintain vigilance at all hours of day and night while interacting with high levels of automation in the space environment, analogous to the air traffic control environment (Projects 2 and 3).

Execution of life science research remote from the principal investigator: Expert systems are not currently available that can compensate for the lack of effective two-way communication between the astronaut-operator and PI during execution of an experiment, the inability to make on-line protocol changes based on in-flight observations, and the absence of effective refresher training during long-duration missions (Project 5).

The five projects in the Human Performance Factors, Sleep and Chronobiology Team addressed each of these deficiencies in the currently existing information. The research is relevant for the round-the-clock work schedules (day, evening and night work) on the International Space Station, the altered sleep/wake schedule on a Mars surface station, or any other situation in which the work-rest schedule is shifted and sleep loss is incurred. It

also has relevance for ground personnel monitoring orbiting crew members who must do so working round-the-clock schedules.

Our overall strategy was based on the recognition that optimizing human performance in space can be best achieved by: (1) understanding the mechanisms by which the space environment affects physiologic systems and man-machine interaction processes contributing to human performance; and (2) development of countermeasures based on these mechanisms. Approaches ranging from the mechanisms of circadian entrainment and sleep regulation to the development of expert systems for the remote execution of life sciences experiments were integrated with the aim of developing countermeasures and testing their efficacy. Integration was achieved by thematic organization around the principal defining characteristics of the space environment, as discussed above. The impact of these aspects of the space environment on astronaut performance was the unifying theme of the Human Performance Factors, Sleep and Chronobiology Team, which sought to fill these knowledge gaps and to maximize effective countermeasures development. The breadth of expertise on this integrated research team provided the capacity to react to additional human performance issues that arose in the course of countermeasure development by the other teams.

The theme of the Human Performance Factors, Sleep and Chronobiology Team was to investigate specific mechanisms responsible for deterioration of human performance in space and to develop effective countermeasures to minimize human error and optimize human performance in the highly automated space environment.

D. MAJOR SCIENTIFIC/TECHNICAL RESULTS AND FINDINGS OF PROGRAMMATIC SIGNIFICANCE

All five projects on the Team as well as the two synergy grants have reported major accomplishments of significance to the program.

Project 1. Circadian entrainment, sleep-wake regulation and neurobehavioral performance during extended duration space flight (PI: Charles A. Czeisler, Ph.D., M.D.).

In the progress report for years one and two we reported the results of twelve 55-day inpatient studies. To date 18 healthy male and female subjects (ages 20-41) were studied and one is in progress. These studies were designed to evaluate: (1) whether entrainment of the human circadian pacemaker will be disturbed when the strength of the environmental light-dark cycle is reduced; (2) that synchronization of the human circadian pacemaker to the 24.6 h day of Mars will be disturbed; and (3) whether this disturbed circadian synchronization to either the 24.0-hour Earth day or the 24.6 hour Mars day will result in disturbed sleep, impaired daytime performance, reduced growth hormone secretion during sleep and inappropriate secretion of the sleep-promoting hormone melatonin during the waking day. To do so, a strict light-dark cycle was used that was comparable to that used in entrainment studies of plants and animals. This effort amounts to 1030 subject test days in the laboratory. Originally we proposed to complete 882 subject test days for FY97-00 and thus have completed more studies than promised. Entrainment was studied in eight subjects scheduled to a 24.0-h sleep-wake and light-dark schedule, i.e., the average solar day of the Earth, as well as in ten subjects scheduled to a 24.6-h sleep-wake schedule and light-dark schedule, i.e. the period of the axial rotation of Mars. The light intensity to which these subjects were exposed was either < 120 lux maximum (~25 lux in the angle of gaze) or < 8 lux maximum (~1.5 lux in the angle of gaze). The preliminary analyses of these data demonstrate that in the < 120-lux condition, 2 out of 2 subjects scheduled to the 24.0 h day and the 3 out of 4 scheduled to the 24.6 h day remained entrained at an appropriate phase angle. Entrainment was assessed on the basis of the plasma melatonin rhythm In contrast, when the strength of the light-dark cycle was reduced to 8 lux, 1 out of 6 subjects scheduled to the 24.0 h day and 6 out of 6 subjects scheduled to the 24.6 h day exhibited misalignment of the sleep-wake cycle and endogenous circadian melatonin rhythm.

Assessment of the intrinsic period based upon melatonin and body temperature data collected during the forced desynchrony segment of this protocol indicate that individual differences in intrinsic period of the pacemaker contribute to the susceptibility to misalignment. Polysomnographic assessment of sleep revealed that circadian phase

misalignment was associated with marked disruption of sleep consolidation and duration. Circadian misalignment and disturbed sleep associated with extended exposure to a light-dark cycle of reduced strength resulted in impaired endocrine and neurobehavioral function. Nighttime human growth hormone and twenty-four hour mean cortisol levels were lower in misaligned versus entrained subjects. With respect to neurobehavioral performance, reaction times were slower, lapses in attention were greater, and cognitive performance was impaired in misaligned versus entrained subjects. These data indicate that: (1) circadian alignment to the 24 hr Earth day appears to be dependent in part on the strength of the light-dark cycle (light intensity); (2) individual differences in the ability to synchronize to the 24-hr Earth day exist and these appear to be dependent in part on the intrinsic period of the circadian pacemaker; (3) circadian misalignment consistently occurs between the human pacemaker and the day length of Mars when the strength of the light-dark cycle is weak or dim, comparable to that aboard the mid-deck of the space shuttle during the Neurolab mission; (4) circadian misalignment the related sleep disruption has negative consequences on endocrine and neurobehavioral function; The results of this research have important implications for understanding operationally relevant limitations on the ability of the human circadian timing system to adapt to the space environment, particularly during extended duration missions such as the planned manned mission to Mars.

Project 2. Countermeasures to neurobehavioral deficits from cumulative partial sleep deprivation during space flight (PI: David F. Dinges, Ph.D.).

Data acquisition in this project has been completed. A total of 1,783 subjects were initially interviewed; of these, 198 (11%) were qualified at interview and underwent extensive laboratory screening, and 106 were ultimately empanelled in a 14-day laboratory protocol. A total N = 91 subjects completed the 14-day protocol, which resulted in a grand total of 1,344 days in which healthy male and female subjects (mean age = 30 yr) lived in small-group confinement, in isolation, at low light, and with controlled activity and diet, intensive physiological monitoring, and quasi-continuous performance demands. This final completed sample size met the target goal of N = 90 subjects needed to produce response surface experimental maps for hypothesis testing.

The first major discovery from the experiment was that subjects were able to achieve significant levels of physiological sleep in both anchor (nocturnal) and nap (diurnal) sleep opportunities across the 10-days of restriction, regardless of the time in bed allowed for sleep. Even daytime nap opportunities as brief as 0.4 hr (24 minutes) consistently resulted in physiological sleep. This indicates that the diverse range of restricted anchor + nap sleep durations tested in this protocol will likely result in sleep if used by astronauts. Response surface model (RSM) development and hypothesis testing on the large set of neurobehavioral and physiological outcomes are currently underway. Random coefficients regression models are being used to estimate subject-specific mean decrements associated with cumulative exposure to the 18 chronic sleep ration protocols for primary performance, subjective, and physiological variables. These subject-specific slopes are being used in response surface modeling in order to identify optimal nocturnal anchor sleep + daytime nap combinations that minimize adverse effects on neurobehavioral and physiological functions from chronic sleep restriction. The spatial location of optimal solutions are also being graphically illustrated by plotting the expected slope as a function of the response surface model. All response surface models being evaluated include the following factors when attempting to optimize the benefits of reduced sleep opportunities: (1) nocturnal anchor sleep duration, (2) midday nap sleep duration, (3) baseline individual differences, (4) age and (5) gender. An anchor sleep by nap interaction is first assessed, and if not significant, a model that includes squared terms for nap duration and age is evaluated. These latter two are then removed one-at-a-time if not significant. Thus, when there is no interaction the final RSM is selected using either the actual response value (i.e., two-stage regression with intercept) or the change from baseline value (i.e., two-stage regression without intercept). In general, we have found that objective performance variables are best evaluated using the actual response/with intercept first stage model, while subjective response variables are best evaluated using the change from baseline/no intercept first stage models.

Preliminary analyses developing RSMs for psychomotor vigilance task (PVT) performance lapses (i.e., an objective measure of neurobehavioral performance) and Karolinska Sleepiness Scale (KSS) scores (i.e., a subjective

measure of sleepiness) have been completed in n = 86 (of 90) subjects. Findings from the best-fitting RSM for PVT lapses found no significant contribution from age or gender, but clearly demonstrated a significant difference in neurobehavioral performance between nocturnal anchor sleep durations. As daily anchor sleep duration was decreased from 8.2 hr, to 6.2 hr, to 5.2 hr, to 4.2 hr time in bed each night, there were systematic increases in PVT performance lapses. In parallel, as the duration of daily time in bed for a nap decreased from 2.4 hr, to 2.0 hr, to 1.6 hr, to 1.2 hr, to 0.8 hr, to 0.4 hr, to 0.0 hr, PVT performance lapses increased. There was no interaction between anchor and nap sleep durations. Similarly, the response surface map for the KSS scores demonstrated a significant difference in subjective sleepiness levels between the anchor sleep durations, with a greater degree of cumulative subjective sleepiness reported as both anchor and nap sleep durations shortened. Thus, in preliminary analyses both a representative performance metric (PVT) and a representative subjective metric (KSS) revealed that systematic reductions in daily total time in bed per 24 hours across the 18 sleep-wake schedule conditions resulted in does-response cumulative deficit slopes. This finding confirms and extends evidence that daily sleep durations in the range typically experienced by astronauts (i.e., 4-6 hr/day) result in cumulative neurobehavioral deficits. Ongoing RSM analyses and hypothesis testing are underway on a wide range of additional neurobehavioral, endocrine and physiological variables. Analyses are also being undertaken on the actual amount and timing of physiological sleep obtained in each of the 18 chronic sleep-wake conditions relative to the cumulative slopes (across days) in neurobehavioral and physiological outcomes. The outcome of these analyses will determine whether there are restricted sleep conditions involving naps that minimize cumulative deficits, or whether it is the total amount of sleep per 24-hr period—regardless of how it is obtained via anchor and/or nap sleeps—that determines alertness and performance. Either outcome will enhance our ability to provide astronauts with effective countermeasures for maintaining waking performance capability during long-duration space flight.

Project 3. Quantitative EEG monitoring of vigilance: Effect of sleep deprivation, circadian phase and sympathetic activation (PI: Derk Jan-Dijk, Ph.D.).

Twelve subjects have now completed the primary 12-day research protocol and the data acquisition is complete. Analyses of these and other data have yielded a number of important results and discoveries.

1. The spectral composition of the EEG during wakefulness exhibits pronounced and predictable changes during a 24-h period of sustained wakefulness.
2. The changes associated with sleep loss are most pronounced in EEGs derived from frontal areas of the brain, and in particular so in the delta and theta frequencies, both during wakefulness and during sleep.
3. Changes in alertness and psychomotor vigilance correlate with changes in EEG power density in the delta and theta frequencies in frontal derivations.
4. The incidence of slow eye movements during wakefulness increases during sleep loss and correlates with changes in alertness and psychomotor vigilance. This correlation is so tight that inter-individual differences in the time course of the incidence of slow eye movements closely resemble the inter-individual differences in the time course of neurobehavioral performance during a 24-h episode of sustained wakefulness.
5. The circadian pacemaker modulates the incidence of slow eye movements as well as the spectral composition of the EEG during wakefulness
6. Light-induced changes in the amplitude of the circadian pacemaker and associated changes in the amplitude of the circadian modulation of alertness are associated with changes in the amplitude of the circadian modulation of the incidence of slow eye movements
7. Light-induced acute changes in alertness are associated with acute changes in the EEG and the incidence of slow eye movements during wakefulness
8. Posture modulates the effects of sleep loss and circadian phase on neurobehavioral performance as assessed by the psychomotor vigilance test such that the detrimental effects of sleep loss/circadian phase are more pronounced when subjects are in a supine posture during 40-h of wakefulness.
9. The incidence of slow-eye movements during a drowsiness test predicts performance on a psychomotor vigilance task conducted one hour later.

These new findings establish a close and robust association of frontal EEG and EOG parameters with changes in neurobehavioral performance in a variety of protocols in which sleep homeostasis and circadian rhythmicity were manipulated. These data indicate that EEG/EOG based on-line monitoring of alertness/performance can serve as a practical and attainable tool to predict and prevent critical decrements in performance and alertness, without the need to conduct time consuming tests of neurobehavioral performance. To further develop and validate these methods experiments in which neurobehavioral performance decrements are induced by cumulative sleep loss, such as reported for space flight, are needed.

Project 4. On-line analysis of physiologic and neurobehavioral variables during long-duration space missions (PI: Emery N. Brown, M.D., Ph.D.).

At the outset of Year 2, two changes were made in the research plan. It was decided to develop the dynamic assessments of circadian phase on the basis of forced desynchrony studies (28-h sleep-wake and light-dark cycle), rather than on the basis of the data collected during the 24-h sleep-wake and light-dark cycle experiments. In addition it was decided to use circadian phase data to first adapt the Jewett and Kronauer model to predict average performance rather than performance of individuals. To date, seven control subjects have completed the forced desynchrony segment of the protocol of project 1. Dynamic phase and amplitude assessments were conducted on 6 of those 7 subjects. For 5 of the 6 subjects it was possible to use the differential equation model to decompose each core-temperature series into its circadian, forced desynchrony and thermoregulatory components and reliable dynamic assessments of circadian phase could be made as early as 10 days after the start of the forced desynchrony. Model fitting for the 6th subject has proved difficult because of numerical instability problems.

All the models were fit to the core-temperature data using maximum likelihood based on a Kalman filter and Runge-Kutta procedure imbedded in a Newton's procedure. The Newton's method did not always perform well because the parameter space has many local minima and flat regions. In these cases, the algorithm can fail to find the optimal parameter estimates because of poorly defined second derivatives. Therefore an algorithm for using a stochastic method to find the best parameter estimates for the model based on a genetic algorithm was developed and implemented. In addition, this project has been developing a more physiologically consistent model of the human circadian system that can account for the significant second harmonic as observed in core temperature data. Finally, for 16 to 23 days this project has been able to make dynamic assessments of circadian phase from core body temperature data and combine those estimates with subjects' sleep-wake history in order to predict average performance and subjective alertness in a single subject. This work convincingly establishes the concept that, given circadian phase information from core-temperature data, the performance and subjective alertness models are able to predict average performance and alertness for an actual subject.

The neurobehavioral models have been programmed into a user-friendly software package so that given a subject's sleep/wake history predictions of alertness and performance can easily be made.

We have developed and implemented a model to reliably analyze growth hormone from experimental data. We are using the algorithm to analyze growth hormone data which will be collected as part of Project 2. A manuscript on this work is under preparation.

We have published two major manuscripts on our core-temperature modeling: Brown and Luithardt (1999) and Brown et al. (2000). A second manuscript on our homone modeling has been submitted and a manuscript detailing the core-temperature and neurobehavioral analysis is under consideration.

Project 5. Ground based study and evaluation of Principal Investigator-in-a-Box (PI: Lawrence Young, D.Sc.).

The project investigates the ability of a real-time expert system to improve performance and reduce the time needed to detect and diagnose faults in a realistic space life science experiment that monitors sleep. Results from the study indicate that an expert system can be used for fault management in a space life science experiment. However, its utility depends, at least in part, on training and the user's computer literacy.

The feasibility of the PI-in-a-Box concept has been shown in ground studies as well, confirming the favorable experience with it in space. With appropriate training, [PI] reduced time to detect and correctly troubleshoot faults in a sleep instrumentation setup. We found that by observing the reliability of the indicator lights, [PI] was helpful for subjects on Day 1, and was a hindrance for them on Day 2. There were also fewer undetected anomalies and undiagnosed faults with [PI] than without it. Phase 2 of the study demonstrated a beneficial effect of [PI] in reducing anomaly troubleshooting time. Questionnaires showed that most subjects preferred monitoring the [PI] indicator lights *while* monitoring waveforms, rather than monitoring the waveforms alone. On one hand, [PI] did not improve the reliability of detection, since subjects were not any more correct in their anomaly detection with [PI] than without it. On the other hand [PI] did even out performance by reducing the chance of an undiagnosed fault, and by helping subjects with different tasks based on their experience level. It was shown that [PI]'s indicator lights only needed to be 40% reliable for subjects to achieve optimum performance, which shows its flexibility. [PI] correctly detected the anomalous signal for up to 85% of the time [2]. There was no difference in fault management performance between genders.

Synergy Project 1. Immunomodulatory Consequences of Sustained Partial Sleep Deprivation: *In vitro* and *In vivo* Indices (PI: Janet M. Mullington, Ph.D.).

During this one-year, project plasma samples were obtained and analyzed from 10 subjects participating in the 8.2/0 and 4.2/0 anchor-sleep/nap sleep condition of the protocol employed in Project 2. Analyses of white blood cells in the partial sleep deprivation condition (4.2/0) yielded results consistent with those of total sleep deprivation, i.e. lymphocyte and monocyte numbers rose from baseline. All subjects had increased platelet numbers after 10 nights of PSD. Cytokine measurements of IL-1 receptor antagonist and high sensitivity C-reactive protein, a marker of the acute phase reaction in inflammation, were significanly elevated by the 10^{th} day of PSD.

This project also evaluated the presence of latent viruses in PBMC DNA extracted from blood collected from the subjects in the partial sleep deprivation condition. All samples were negative for BKV, JCV, and SV40 DNA sequences. Analyses for EBV in the cellular DNA of white blood cells failed to find changes attributable to the sleep loss condition. DNA extracted from sedimented urine pellets derived from urine collected from subjects were tested for the presence of BKV, JCV, and SV40. Only one subject was positive for JCV at both baseline and following 10 nights of PSD. No SV40 or BKV was detected in any urine sample.

In addition to the neuroimmune factors mentioned above, growth factor IGF-1 increased during sustained partial sleep deprivation.

Synergy Project 2. Acute total and chronic partial sleep deprivation: Effects on neurobehavioral function, waking EEG and the renin-angiotensin system. (PI: Derk-Jan Dijk, Ph.D.). This synergy grant was awarded in April of 1998. The progress that has since been made includes the purchase of the VITAPORT digital sleep recorder and modifying details of the experimental design. In addition, neurobehavioral data and EEG/EOG data have been collected for two subjects who participated in a head down tilt bed rest study (PI: Dr. G.H. Williams) of the cardiovascular team. One major goal of this synergy grant was to assess the effects of sleep loss on neurobehavioral performance and its electrophysiologic correlates while subjects are in a head down tilt bed rest study. A HDT study can be considered a first step in the evaluation of the accuracy/effectiveness of a countermeasure developed under earth conditions but designed for the space environment. The preliminary analyses indicate that slow eye movements are present during the scheduled wake episodes of subjects even though these subjects had an 8 h sleep opportunity during each day of the protocol. This may indicate that the continuous bedrest

HDT condition induces daytime sleepiness in subjects who can sleep for 8 h each night.

E. INTEGRATION OF PROJECTS INTO COHESIVE PROGRAM

During the project period, the projects were well integrated into a cohesive program, both conceptually and methodologically. In addition to complementing each other conceptually through emphasis on the relative contributions and interactions of the endogenous circadian pacemaker and the homeostatic drive for sleep, there were active and ongoing collaborations across laboratories as illustrated by the following. Neurobehavioral tests developed in Project 2 (e.g., psychomotor vigilance test; probed recall memory test; time estimation task) were integrated into Projects 1 and 3, providing common neurobehavioral performance and subjective metrics across protocols. This permitted direct comparisons of the results from the different experiments, and provided a broad, consistent information set for input into the mathematical model that was developed in Project 4. Projects 1, 2, and 3 also performed comparable physiological monitoring, which included assessment of circadian phase using core body temperature; assessment of sleep using standard polysomnography; and assessment of waking alertness using waking EEG during the Karolinska Drowsiness Test (gaze fixation task). Finally, Project 5 on Principal Investigator-in-a-Box used data collected by the team leader during flight experiments on STS-90 and STS-95. Synergy Project 1 on Immunomodulatory Consequences of Sustained Partial Sleep Deprivation used data from Project 2. Project 3 described direct effects of different illuminances on alertness as well as the EEG and EOG, demonstrating that light, beside its role as synchronizer of the circadian system as investigated in Project 1, also exerts major effects on alertness and its ocular and electroencephalographic correlates. In short, a major strength of the Team has been that the Projects provided a cohesive program for analysis of the sleep and circadian factors that are important in human performance and vigilance. These projects also provided a strong foundation for the development of techniques that should be effective in monitoring and improving performance and vigilance during long space flights. The success of the integration of the projects into a cohesive program was evident at the First Biennial Space Biomedical Investigator's Workshop sponsored by NASA and the NSBRI. During this workshop all projects were presented in the Sleep/Circadian Rhythm session chaired by Drs. Dinges and Czeisler.

F. INTERACTION & SYNERGY WITH OTHER TEAMS

The successful efforts by Dr. David Dinges (PI of Project 2) to obtain approval from the University of Pennsylvania IRB and the U.S. Food and Drug Administration to use of the Carmeda ConFlo™ system for continuous blood sampling may contribute significantly to the implementation of such a blood sampling system by other teams and in other experiments and thereby contribute to the interaction and synergy with other teams. Application of the growth hormone and melatonin studies to the space environment will be facilitated by development of the mini-mass spectrometer for hormone measurements by the Instrumentation Research and Development Team. The growth hormone studies may have direct implications both for understanding the extent to which sleep restriction and sleep disruption may reduce circulating growth hormone during space flight and for determining the extent to which improved circadian entrainment, coupled with naps of optimal duration, may increase availability of this hormone, of great importance for the Cardiovascular Alterations, Muscle Alterations and Atrophy and Bone Demineralization/Calcium Metabolism teams. Neuroendocrine data can be pooled among the Human Performance Factors team and Cardiovascular, Muscle and Bone teams to create a descriptive database of neuroendocrine parameters under different space simulation and ground-based study conditions.

The quantitative EEG/EOG monitoring of vigilance project contributes to all teams in which neurobehavioral function is assessed. The AI project (PI in a Box) contributes to the efficient conduct of life-sciences experiments in space for each of the other seven team areas. Finally, on-line time series analysis will support evaluation of physiologic data collected from novel technologies developed by Instrumentation Research and Development Team.

Perhaps the best evidence for the developing synergy between the Human Performance Factors, Sleep and Chronobiology Team (HPFSC) and other NSBRI Teams is the fact that the two synergy projects submitted from the HPFSC Team have successfully collected data across teams. Synergy Project 1, entitled "Immunomodulatory Consequences of Sustained Partial Sleep Deprivation: *In vitro* and *In vivo* Indices" (PI: Janet M. Mullington, Ph.D.), synergized the aims of three NSBRI research teams (Human Performance Factors Team; Immunology Team; and Muscle Team) by investigating the neuroendocrine and neuroimmune modulation of sustained partial sleep deprivation (HPFSC Team Project 2) to test the influence of cumulative sleep loss on the reactivation of latent viruses, and the delayed hypersensitivity response to cutaneously applied antigen testing. Synergy Project 2, entitled "Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and the Renin-Angiotensin System" (PI: Derk-Jan Dijk, Ph.D.), synergized the aims of two NSBRI research teams (Human Performance Factors Team; and Cardiovascular Team) by investigating neurobehavioral function and waking EEG in the research protocols of the renal-cardio endocrine project and renin-angiotensin and cardiac function in the research protocol of the quantitative EEG and waking neurobehavioral function project. Quantitative analyses of the effects of sleep deprivation and posture on the electrocardiogram have been implemented in the research protocol of Project 2.

Additional contributions to the synergy with other teams includes the contribution of Project 2 of more than 1,000 ml of plasma to Dr. William Shearer, Head of the NSBRI Immunology, Infection and Hematology Team, for the study of key regulatory cytokines and soluble cytokine receptors in relation to severe acute total sleep deprivation (88 hr awake), partial sleep deprivation, chronic low-dose caffeine intake, and circadian rhythmicity.

G. CONTRIBUTION OF PROGRAM TO NSBRI MISSION

The research program of the Human Performance Factors, Sleep and Chronobiology Team was a goal-directed research program that has provided an integrated contribution to the overall NSBRI mission and has addressed the Institute's Aims and Objectives by:

(1) Designing, implementing, and validating effective countermeasures to address the biological and environmental impediments to long-term human space flight. These countermeasure relate to the conditions, consequences and requirements of the space environment such as absence of geophysical cycles and associated disrupted circadian entrainment (Projects 1, 4); microgravity and associated sleep loss/disruption and reduced growth hormone secretion (Projects 1, 2, 3); the high level of automation that requires continuous vigilance and supervision (Projects 3); and the remote inaccessible location of space that requires execution of protocols without PIs (Project 5). Countermeasures include Intermittent Bright Light and Melatonin (Project 1), Preemptive Naps and Sleep Management (Project 2), Quantitative EEG/EOG Monitoring for early detection of performance decrements (Project 3), Early Detection of Circadian Physiologic Anomalies (Project 4), and Expert Systems for Scientific Experiments (Project 5).

(2) Defining the whole-organism integrated-physiological and neurobehavioral responses that ultimately determine these impediments and designing novel countermeasures based on these responses. These responses have included loss of circadian entrainment (Projects 1, 4); sleep-loss and sleep disruption (Projects 2, 3), altered sympathetic activation (Project 3), and unsupervised decision processes (Project 5).

(3) Establishing support technologies to maximize human performance in space and to reduce the probability of human performance failure. These technologies have include novel intermittent bright light application protocols (Project 1); new rest-activity and sleep scheduling techniques (Project 2); quantitative on-line vigilance monitoring techniques (Project 3); novel algorithms for near on-line assessment of circadian phase and amplitude (Project 4); and expert system technologies that will aide execution of scientific experiments in the absence of the PI (Project 5).

(4) Transferring and disseminating the advances in knowledge and technology acquired to populations on earth in which performance is jeopardized. These situations and populations include shift workers and older people in whom circadian entrainment is jeopardized and sleep disruption is prevalent (Projects 1, 2, 3, 4) and the biomedical/hospital and science environment in which decision making is often required while highly experienced experts are unavailable.

(5) Providing training of new scientists in the space life sciences by recruiting young scientists for active participation in the proposed ground-based research program and by mentoring these young scientists over the next three years. Ten post-doctoral trainees have been supported by the proposed program in order to assist in the data collection, analysis and publication of the ground-based research projects. Four further post-doctoral trainees have contributed to the projects without receiving financial support from the program. This had ensured that these trainees acquired a thorough knowledge of the human performance factors, sleep and chronobiology research related to long duration space missions. We have also broadened participation in the activities of the NSBRI to extend beyond the consortium by providing an opportunity for a non-consortium institution (University of Pennsylvania, Project 2), to contribute to the evolution of the research theme of our team. The investigators on the team have a long established track record of disseminating the results of their research findings to work-shops, cross-disciplinary symposia and publications. Finally, the investigators on the Human Performance Factor, Sleep and Chronobiology research team have a history of demonstrated active collaboration with NASA investigators at JSC, ARC and other NASA facilities, which continued throughout the third year.

II. RISK REDUCTION ACHIEVED BY PROGRAM

Provided below is a summary of the risk reduction relative to the NASA's Critical Research Path achieved by each of the projects within the Program

Results from Project 1 experiments carried out since October 1, 1997 suggest that exposure to a light-dark cycle of dim room light may be sufficient to maintain circadian entrainment to a 24.0 hr day in the subjects tested, whereas it was not sufficient to maintain entrainment to the 24.6 hr day in all of the subjects tested on this schedule. As noted above, analysis of the sleep, endocrine and neurobehavioral performance data indicates that the subjects who were not entrained at an appropriate phase angle suffered from sleep disturbance, altered endocrine function and impaired neurobehavioral performance as compared with the subjects entrained at an appropriate phase angle. The analyses also indicate that besides the strength of the light-dark cycle, the individual intrinsic period is a determinant of entrainment. This finding has important implications for understanding entrainment to altered light-dark and sleep-wake schedules. The results of our studies demonstrate that circadian maladaptation to the 24.6 h day results in sleep disturbance, diminished nocturnal growth hormone secretion and impaired vigilance and cognitive performance. Thus, the development and evaluation of appropriate countermeasures to facilitate adaptation to the 24.6 h Martian day are critical to the success of both a Mars surface station and a Mars orbiting platform. The results of this research have important implications for understanding operationally relevant limitations on the ability of the human circadian timing system to adapt to the space environment, particularly during extended duration missions such as a manned mission to Mars.

CRITICAL PATH questions for the NSBRI Human Performance Factors, Sleep and Chronobiology Area
Performance failure because of sleep and circadian rhythm problems (19)
Description
Human performance failure due to disruption of circadian phase, amplitude, period, or entertainment, and/or human performance failure due to acute or chronic degradation of sleep quality or quantity.
Risk Factors *Circadian Rhythms, Sleep Disorders,*
Risk Type II

Risk Rank (within Discipline Risk Area) 2

6.05 What are the acute and long-term effects of exposure to the space environment on biological rhythmicity, on sleep architecture, quality, and quantity, and their relationship to performance capability?

This critical path question is addressed in Project 2 through extensive examination of the neurobehavioral and physiological effects of chronic daily sleep durations in the range commonly experienced by astronauts in space flight (i.e., averages between 4 hr sleep per day and 6.5 hr sleep per day (mean = 6.0 hr sleep per day). In spite of recommendations to astronauts to sleep 6 to 8 hours per day while in orbit, there is considerable evidence that during space flight astronauts and cosmonauts typically sleep for shorter periods relative to when on the ground (e.g., 6.0 hr per day in space compared to 7.9 hr per day on Earth), resulting in chronic partial sleep deprivation. In the current project we track the changes in objective and subjective neurobehavioral functioning, mood, circadian rhythms, endocrine function, physiological alertness, and sleep quality and structure across 10 days of sleep restricted to each of 18 different durations, spanning the dynamic range of sleep durations consistently observed in astronauts during space flight. Therefore the project will clearly establish the effects of exposure to sleep restriction of the kind experienced in space flight. Thus far results from the project confirm and extend evidence that daily sleep durations in the range typically experienced by astronauts (i.e., 4-6 hr/day) result in cumulative neurobehavioral deficits.

6.06 Which countermeasure or combination of behavioral and physiological countermeasures will optimally mitigate specific performance problems associated with sleep loss and circadian disturbances during a Mars mission?

This critical path question is directly addressed in Project 2 through examination of napping as a potential countermeasure during a period of chronically reduced nocturnal anchor sleep. Although there is evidence that the less sleep obtained, the greater the waking deficits, experiments have found that for acute periods, supplementing a reduced anchor sleep period with a nap has the potential to enhance performance, due to the exponential recovery of neurobehavioral functions relative to sleep duration. There is a critical deficiency in knowledge of the effects of varying combinations of anchor sleep and nap durations that will yield the most efficient return of performance per unit time invested in sleep. The primary aim of this project is to meet this critical deficiency through use of a response surface experimental paradigm, testing in a dose-response manner 18 different combinations of anchor sleep and nap sleep durations for the purpose of establishing how to most effectively limit the cumulative effects of chronic sleep restriction in space operations. The preliminary response surface maps derived from the current dose-response protocol indicate that total sleep time per 24 hr is the prime determinant of cumulative neurobehavioral responses, and that combining a restricted nocturnal anchor sleep with a midday nap may attenuate cumulative deterioration in performance resulting from sleep restriction in the range observed during space flight.

6.07 What are the long-term effects of countermeasures employed to mitigate performance problems with sleep loss and circadian disturbances during a Mars mission?

This critical path question is addressed in Project 2 through examination of whether or not subjects can consistently actually achieve restorative physiological sleep, and the cumulative benefits of that sleep for neurobehavioral and physiological functions, when exposed to novel sleep-wake schedules involving nocturnal anchor and diurnal nap sleep. In addition, the potentially long-term adverse effects on performance immediately after awakening (i.e., sleep inertia effects) from nocturnal anchor sleep and daytime naps is being assessed to ensure that the acute disadvantages of novel sleep-wake schedules do not outweigh the longer-term advantages (i.e., ensuring that the costs of any "optimal" schedule are thoroughly understood).

6.08 What are the best methods for monitoring the status of sleep and circadian functioning and for assessing the effects of sleep loss and circadian dysrhythmia that are also portable and non-intrusive in the spaceflight environment?

This critical path question is addressed in Project 2 through repeated (every 2 hours during wake) examination of neurobehavioral functioning using the neurobehavioral assessment battery developed in our laboratory. Neurobehavioral metrics of performance capability make up some of the key dependent variables in the experiment. Standardized measures of performance are contained in the computerized neurobehavioral assessment battery we developed and validated in sleep deprivation and circadian experiments. The NAB was developed to be an optimally sensitive suite of neurobehavioral measures (assays) that could be used in studies of sleep loss and circadian rhythms requiring frequent and repeated assessments. It contains multidimensional aspects of performance and activation and the most sensitive and reliable tests of performance impairment from sleepiness that we have been able to identify in the past 20 years. The NAB performance tasks tap many of the neurocognitive functions subserved by brain regions such as the prefrontal cortex, cingulate gyrus, and thalamus that have been found to be affected by total sleep loss in neuroimaging studies with positron emission tomography. One of the most sensitive measures in the neurobehavioral assessment battery is the psychomotor vigilance task (PVT), which is extensively validated to reflect endogenous circadian variation and both acute and chronic sleep loss. The PVT is portable; devoid of a practice curve; and free of acquired skill bias (aptitude, education), making it exceptionally well suited for brief repeated assessment of neurobehavioral function in space flight. The PVT is currently being examined by a working group at Johnson Space Center (Dr. Chris Flynn) as a prototypical tool for fatigue assessment in space flight.

6.21 What mathematical and experimental models best predict performance problems associated with sleep-wake and work history and circadian rhythm status, and also provide guidelines for successful countermeasure strategies?

This critical path question is addressed in Project 2 by providing neurobehavioral data results from the Project to Drs. Jewett and Kronauer at Harvard for inclusion in the development of a biomathematical model of control of neurobehavioral functions by circadian rhythmicity and sleep homeostasis. Dr. Jewett has developed a preliminary model of psychomotor vigilance performance using data collected in the studies in both Dr. Czeisler's (Project 1) and Dr. Dinges' (Project 2) laboratories. This model is able to accurately predict the increase in the number of lapses of attention that occurs during sleep deprivation.

In project 3, the research conducted in the current grant period is aimed at the development of countermeasures for Human performance failure because of sleep and circadian rhythm problems [Risk 19, Critical Road Map http://criticalpath.jsc.nasa.gov]. In particular our research relates to the critical questions 6.08 (What are the best methods for monitoring the status of sleep and circadian functioning and for assessing the effects of sleep loss and circadian dysrhythmia that are also portable and non-intrusive in the spaceflight environment?) and 6.21 (What mathematical and experimental models best predict performance problems associated with sleep-wake and work history and circadian rhythm status, and also provide guidelines for successful countermeasure strategies?). In addition our research is relevant to critical question 6.05 (What are the acute and long-term effects of exposure to the space environment on biological rhythmicity, on sleep architecture, quality, and quantity, and their relationship to performance capability?) and 6.06 (Which countermeasure or combination of behavioral and physiological countermeasures will optimally mitigate specific performance problems associated with sleep loss and circadian disturbances during a Mars mission?).

In Project 4, findings to date support the approach of decomposing the core-temperature rhythm into its circadian, activity and thermoregulatory components under the current protocol (Project 1), which simulates the light-dark conditions of long-duration space missions. The van der Pol oscillator representation of the circadian signal, as suggested by Kronauer and modified by Jewett, provides a very rich framework for analyzing the dynamic structure of the circadian system. However, the predictive value of this model may be further enhanced by modifications as described in Project 4. The most important feature of this is the pacemaker's response to light input as observed in core-temperature data. These two models will continue to be used as the basis for representing these

parts of the data. The changes in the statistical models and methods applied will be implemented for each model formulation that offers an important improvement in the ability of the model to describe the data. With each new formulation of the model, its ability to predict accurately circadian phase and amplitude will be assessed. Each new representation of the model will be used to generate circadian phase and amplitude inputs that will allow us to predict performance based on the Jewett and Kronauer neurobehavioral performance model, which will be updated by neurobehavioral data in Projects 1, 2 and 3. The new growth hormone model has major implications for being able to analyze this hormone both for the purposes of biomedical research in space as well as in standard medical investigations.

For project 5, space experiences with computer decision aids for astronaut scientists have all been demonstrations, rather than formal experiments with testable hypotheses. The drive to develop useable new technology in feasible, cost-effective ways outweighed the scientific need to fly placebo devices as controls for experiments. (These devices would have contributed nothing to the ongoing experiments, would have consumed valuable space resources, and so were considered unessential/dispensable.) Our study performed thorough ground tests to evaluate the efficacy of our expert system for assisting astronauts in the Space Station era. The diagnostic aids, experimental scheduler, and interesting data monitor were shown to be beneficial for carrying out space experiments. Each of the tools developed throughout the history of [PI] – from STS-40 and STS-58 through STS-90 and STS-95 - can be applied to experiments aboard ISS. Autonomous systems are already being implemented in the ISS, and having a software with embedded knowledge such as [PI] will ensure the scientific and operational success of a mission. These developments could reduce the chance of error caused by human-system interface problems, a concern outlined in section 6.09 of the NASA Critical Path Roadmap.

The development of [PI] can be applied to earth-based domains too. Subjects could be helped by an intelligent fault management system for diagnostics and repairs. Earth-based space research includes projects such as the BIOPLEX. Autonomous fault management systems are already being used for this testbed of life support systems. The results of human behavior in a fault management situation, such as in this ground study, could lead to better designs for the interface of such systems. Other earth-based applications include home sleep monitoring. Patients or caregivers who are not familiar with sleep instrumentation can use a diagnostic engine to help them detect and repair failures, without data being lost. Therefore, the concept of embedding the knowledge in an autonomous system in the spirit of [PI] can benefit technology on earth.

Synergy Project 1 has successfully evaluated some immunological consequences of chronic partial sleep deprivation. These findings underscore the multitude of consequences of sleep loss, which has been reported during space flight. The findings also point to the necessity of multidisciplinary assessments and evaluation of any sleep-wake schedule as well as the necessity of a multidisciplinary approach in the evaluation of the effectiveness of countermeasures.

Relates to 6.05, 6.06, 6.07: Problem identification: Early immune system dysregulation in long duration space travel. Implications for future countermeasure studies.

This synergy project brought together areas of importance to the Immunology, Infection and Hematology Team together with the Muscle Alterations and Atrophy Team, and the Human Performance Factors, Sleep and Chronobiology Team. This synergistic pilot study was aimed at identifying a potential consequence of a known problem experienced in the space environment: sustained chronic sleep restriction. We used the two extreme conditions of project 2 (Dinges PI) in order to better contrast the effects of this cumulative sleep deficit on immune parameters and growth factors. The preliminary findings of IL-1 receptor antagonist and CRP suggest alterations in inflammatory processes introduced by sleep loss, thus identifying a previously unknown consequence of sustained sleep restriction. These findings are important and warrant confirmation and extension. One obvious countermeasure that needs to be tested is the potential efficacy of napping in immune protection and preservation of the

inflammatory response during long-duration space flight. Since we have found evidence for altered IGF-I we will continue to monitor this growth factor in future protocols. IGF-I may simply reflect increased drive for slow wave sleep (Prinz et al., 1995), but growth factors are also involved in immune regulation, and therefore we will continue to monitor this association in future studies of sleep loss.

The consequence of early immune system dysregulation could be very serious for long-term space flight in that an ineffective or over-reactive early immune system host response could seriously affect health maintenance or illness recovery of the crewmembers, and thereby jeopardize mission success.

Synergy project 2 has successfully implemented the recording of EEG/EOG as well as the assessment of neurobehavioral performance during a continuous bedrest study (head down tilt). The preliminary data which indicate that slow eye movements are prevalent even during a normal 16 h waking episode may imply that these conditions are indeed very conducive for sleepiness. Further investigation of the time course of alertness and neurobehavioral performance in this protocol and comparison of these data to data on neurobehavioral performance and alertness in different conditions will provide insights into the effects of continuous bedrest.

III. APPENDIX

A. BACKGROUND AND SIGNIFICANCE

The success of long-duration manned space flight depends on maintenance of high level human performance and vigilance while monitoring sophisticated instrumentation, and is attended by close public scrutiny and a high cost of failure. On earth, human error is the most common root cause of accidents in technology-rich environments (Reason 1990; Wiener, 1993; Senders and Moray 1991; Dinges 1995), such as commercial aviation, where 68% of accidents are attributable to performance errors by cockpit crews (Nagel 1988). In space, the contribution of human performance factors to mission success is even greater, since fundamental aspects of the space environment compromise physiologic systems critically involved in human performance (Graeber 1988; Dinges 1995). Essential characteristics of the space environment are absence of geophysical cycles, microgravity, high level of automation and remote, inaccessible location. Such conditions are know to affect physiologic, behavioral and cognitive processes critically involved in human performance. These physiologic, behavioral and cognitive processes include circadian entrainment, sleep homeostasis, continuous vigilance and decision processes.

Circadian Entrainment and Sleep Homeostasis

There is extensive anecdotal (Santy *et al.* 1988) and recent objective evidence (Gundel *et al.* 1997) that astronaut sleep is chronically restricted in space flight to averages between 4 and 6 hours/day. This is a potentially serious problem for extended duration space flight because sleep is among the most fundamental determinates of waking performance, and frequent restriction of sleep during space flight poses a risk to the neurobehavioral and operational capability of astronauts. The mechanism through which this risk emerges is the development of cumulative homeostatic pressure for sleep across consecutive days of limited sleep (Carskadon and Dement 1981; Carskadon and Roth 1991; Dinges *et al.* 1997). Research to date has established that cumulative sleep debt can emerge with or without a stable circadian phase entrainment (Rosekind et al. 1994; Dinges *et al.* 1997); the "debt" can disrupt the same waking neurobehavioral performance parameters affected by acute total sleep loss (Wilkinson 1969; Carskadon and Roth 1991; Dinges and Kribbs 1991; Dinges 1992; Dinges *et al.* 1997); and the physiological sleepiness and performance deficits engendered by the sleep debt can progressively worsen (i.e., accumulate) across consecutive days of sleep restriction (Carskadon and Dement 1981; Herscovitch and Broughton 1981; Rosenthal *et al.* 1993; Dinges *et al.* 1997). In fact, sleep restricted to 4 - 6 hr for 7 - 14 days results in 4- to 10-fold increases in psychomotor vigilance lapses (see Preliminary Results section on Project 2).

To compound the risk to sustained optimal neurobehavioral output essential for success during extended duration space missions, astronauts are exposed to markedly abnormal light-dark cycles. These light-dark cycles have a reduced *Zeitgeber* (German for "time giver" or synchronizer) strength and may be inadequate to maintain the appropriate phase-relationship between the 24-h sleep-wake and work cycle and the endogenous circadian timing system. Circadian misalignment is associated with nocturnal sleep disruption and deterioration of daytime alertness and cognitive performance, and can result in lapses of attention during the extended duty hours sometimes required during space missions. The basis of these changes involves abnormalities in the interaction between processes regulating human performance and sleep. Misalignment of circadian phase and work-sleep schedules can overpower an individual's ability to remain awake and attentive while working at night (Strogatz *et al.* 1986; Rosekind *et al.* 1991; Rutenfranz *et al.* 1972; Tilley *et al.* 1982), which can lead to impaired job performance and higher rates of accidents and injuries (Smith *et al.* 1982; National Commission on Sleep Disorders Research, 1993; Mitler *et al.* 1988).

Most if not all physiologic and behavioral variables exhibit endogenous circadian rhythms. In the absence of environmental synchronizers, the period of these rhythms deviates slightly but significantly from 24-h. On earth, the phase relation between these endogenous circadian rhythms and the 24-h day is maintained by a process called entrainment. In the past two decades, remarkable progress has been made in the understanding of the neuroanatomical structures involved in the generation of endogenous circadian rhythms, the interaction of the endogenous circadian timing system and other regulatory (homeostatic) processes in the regulation of sleep propensity, sleep structure, daytime alertness, and cognitive performance, and the mechanisms of entrainment of the human circadian pacemaker by the light-dark cycle (Czeisler 1995). In fact, it has been found that exposure to bright light and darkness can produce rapid physiologic adaptation of the circadian pacemaker to a single week of night work (Czeisler *et al.* 1990). In addition, bright light can enhance alertness of night workers during their scheduled work shifts (Campbell and Dawson 1990; Dawson and Campbell 1991).

Regulation of sleep, subjective alertness and cognitive performance. The endogenous circadian pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, is a major determinant of sleep propensity, sleep structure and daily variations in subjective alertness and cognitive performance (Duffy *et al.* 1992; Dinges *et al.* 1987; Dijk *et al.* 1992; Johnson *et al.* 1992; Folkard and Åkerstedt 1992; Åkerstedt *et al.* 1982a). In fact, a clinical case report indicates that damage to the rostral SCN can lead to disruption of the temporal pattern of the sleep-wake cycle, body temperature, cognitive and behavioral functioning (Cohen and Albers 1991). Early evidence of a clock mechanism underlying variations in alertness, performance and sleep propensity was derived from long-term sleep deprivation experiments. In these experiments, alertness and performance exhibited rhythmic variations with a period close to 24 hours superimposed on a steady deterioration of alertness and performance, attributable to sleep loss (Fröberg *et al.* 1975a; Fröberg *et al.* 1975b; Fröberg *et al.* 1972; Åkerstedt and Fröberg 1977; Åkerstedt *et al.* 1979). The notion that alertness, performance and sleep propensity are determined by the interaction of these two processes, i.e., a circadian and a sleep-wake dependent process, is widespread and has been formalized in mathematical models (Daan *et al.* 1984; Babkoff *et al.* 1991; Folkard and Åkerstedt 1989; Åkerstedt and Folkard 1990).

Circadian Rhythms in Space. Prominent circadian rhythms exist in many quantifiable behaviors, including sleep tendency (Richardson *et al.* 1982; Carskadon and Dement 1975; Carskadon and Dement 1979; Åkerstedt *et al.* 1982b), vigilance (Beatty *et al.* 1977), alertness (Richardson *et al.* 1982; Czeisler *et al.* 1980), short-term memory (Johnson *et al.* 1992; Babkoff *et al.* 1988; Babkoff *et al.* 1991), and performance (Colquhoun 1981). Recent studies have demonstrated that the endogenous components of the circadian rhythms in these behavioral variables are coupled to the endogenous rhythm in body temperature, such that performance decrements typically occur in the early morning hours, at the time of the trough of the endogenous circadian temperature cycle. These performance decrements are most pronounced in those tasks requiring sustained attention (Wilkinson *et al.* 1966; Dinges *et al.* 1987). Following the trough of the temperature cycle, performance generally improves, even when the subjects have

not had recovery sleep. This indicates that early morning performance decrements are a result of a misalignment of circadian phase together with sleep deprivation. In addition recent evidence suggests that the effects of sleep deprivation and circadian phase are reflected in the spectral composition of the waking EEG and that the effects of sleep deprivation become more pronounced when subjects are kept awake in a supine position, i.e. a situation which has been uses as a model for microgravity (see Background and Preliminary Results Section of Project 3).

As noted above, in addition to the regular circadian variation in the behavioral indices, performance can be further affected by sleep deprivation itself, as occurs in shift workers or persons who travel across time zones. Such sleep deprivation is often compounded by circadian disruption, since both the ability to sleep and the timing and internal organization of sleep vary with circadian phase (Czeisler, 1978; Czeisler *et al.* 1980; Strogatz *et al.* 1986; Strogatz *et al.* 1987). Thus, personnel on night duty schedules often find themselves unable to sleep well during their off-duty hours. Therefore, understanding the mechanisms underlying the resetting of the endogenous circadian oscillator, including the neurochemical basis of rhythmicity and entrainment (Albers *et al.* 1991a; Albers *et al.* 1991b; Albers *et al.* 1984), is essential for developing strategies to combat potentially hazardous decrements in performance due to both circadian phase misalignment and sleep deprivation among personnel who must operate around-the-clock and/or across time zones or are exposed to *zeitgeber* cycles of reduced strength.

Previous studies of astronauts suggest that circadian rhythmicity persists during space flight, but a number of abnormalities in the period and amplitude of various rhythms have been reported. Spectral analysis of data from Gemini astronauts during the flight of GT-5 showed a prominent circadian rhythm in heart rate, with a period of 23.7 hours (Rummel *et al.* 1967). Halberg *et al.* have also reported a persistence of cardiac circadian rhythms in space (Halberg *et al.* 1970). Russian scientists have reported that subjects placed in an antiorthostatic position, designed to simulate weightlessness, showed a mean decrease of 0.20 degrees C in the amplitude of daily oral body temperature (Lkhagva 1980). However, their control subjects were not studied in a supine posture, making it difficult to determine whether or not the -8 degrees head-down tilt lead to this difference. Lkhagva (1984) reported that the axillary temperature of a Salyut-6 crew member decreased significantly compared to baseline and pre-flight measures, and that there was a change in the phase of the maximum of the circadian temperature rhythm during the mission. More recently, Gundel *et al.* have reported that the body temperature rhythm of a single astronaut drifted ~ 1.7 hours later during flight as compared to baseline, even though his sleep-wake schedule during the mission was the same as during baseline (Gundel *et al.* 1993). This was consistent with a phase delay in the astronaut's circadian pattern of alertness. However, recent data gathered on LMS-1 revealed no apparent delay in the circadian temperature rhythm among the four astronauts studied although changes in the circadian temperature waveform were observed (Monk *et al.* 1998).

Finally, Gundel *et al.* have reported core body temperature and alertness data from four cosmonauts during a month-long mission aboard the *Mir* Space Station (Gundel *et al.* 1996). They found that both the body temperature and alertness rhythms were significantly delayed as compared to baseline recordings, consistent with the predictions of Kronauer's mathematical model (Kronauer and Czeisler 1993) and with preliminary data from subjects entrained to a light-dark cycle of reduced strength (see preliminary results in Project 1). Continuous monitoring of ambient light levels aboard LMS-1 has recently confirmed this reduction in *zeitgeber* strength, revealing a 3-5 fold reduction in average daytime light levels on the space shuttle, as compared to ground-based readings (Monk *et al.* 1998). During the recent Neurolab mission light levels were assessed in the three habitable compartments of the spaceshuttle (Czeisler et al. 1999). Whereas on the flight deck light levels were highly variable, ranging from less than 1 lux to close to 80000 lux . In contrast in the mid deck and spacelab light levels were near stable and as low as 9 and 100 lux respectively. These studies suggest that in the space environment, both internal and external synchronization of the endogenous circadian multi-oscillator system in animals and humans is impaired. This may be due to the weakening of the strength of environmental synchronizers that entrain the endogenous circadian pacemaker to a 24-hour period on earth.

A number of studies have been performed on the effects of weightlessness on circadian rhythms in animals. Studies on the *Macaca nemestrina* monkey during Biosatellite III (Hoshizaki *et al.* 1971; Hahn *et al.* 1971) showed that weightlessness did not affect the period of the animal's sleep/wake cycle or arterial blood pressure rhythms. However, the period of rhythms in heart rate, brain and body temperature, and pCO_2 increased to 26 hours and were no longer synchronized with the 24-hour period of the sleep-wake cycle in those animals. By the end of the 9-day flight, an approximately 16-hour phase difference had accumulated between those rhythms (Hoshizaki *et al.* 1971). A joint Soviet/American study involving two Rhesus monkeys focused on three measures to assess circadian rhythmicity: skin temperature, body temperature, and motor activity (Klimovitskiy *et al.* 1987). The team reported that the monkeys became "less homeothermic" under the influence of weightlessness, with core temperature controlled less precisely and more dependent on ambient environmental temperature.

It has been reported in the rat (Fuller 1985) that the phase relationship of the body temperature rhythm to the light/dark cycle was altered between pre-flight and actual flight conditions. In addition, the period of the temperature rhythm in flight was longer than during pre-flight conditions (24.4 $\pm$ 0.3 hours vs. 23.9 $\pm$ 0.2 hours, respectively). Heart rate remained both rhythmic and entrained to the 24-hour light/dark cycle, but its amplitude was considerably depressed from 273 bpm pre-flight to 182 bpm in-flight. Taken together, these studies suggest that in the space environment, both internal and external synchronization of the mammalian circadian pacemaker is impaired. This may be due to the weakening of the strength of environmental synchronizers that entrain the endogenous circadian pacemaker to a 24-hour period on earth. Any such effects will be exacerbated during the long-term exposure to the space environment contemplated aboard the International Space Station.

Sleep in Space. Since the initial observation in 1961 that humans could indeed sleep in space, very few data have been systematically collected on the effect of space travel *per se* on the sleep of astronauts. However, Graeber reviewed 28-years of available data on sleep in space (Graeber 1988) and reports that the sleep of astronauts was often disturbed during early, relatively short flights of the American Mercury and Soviet Vostok series. A combination of factors may have contributed to the reported sleep disruption: the initial excitement of space flight, noise disturbance, the effects of weightlessness and the cramped quarters available for sleep (Graeber 1988). However, even in later Apollo missions, poor sleep and subsequent crew fatigue were reported to have interfered with operational requirements (Berry 1969). On one occasion, the Command Module pilot actually fell asleep while on duty, later resorting to amphetamine use to stay awake (Graeber 1988).

The advent of the space shuttle led to a marked improvement of sleeping conditions for astronauts in space. Shuttle crew members have separate sleeping quarters and an attempt has been made to pre-adapt them to their expected duty schedule in space in the week before launch. Nonetheless, a survey of 58 crew members from nine shuttle missions revealed that most crew members suffered from sleep disruption during their missions and were only able to sleep an average of 6.1 hours per day of flight as compared to 7.9 hours each night on the ground (Santy *et al.* 1988). Crew members report losing an average of 2.2 hours per night on the first and last days of flight (Santy *et al.* 1988).

When sleep restriction continues for more than a few consecutive days, a "sleep debt" accrues, leading to the development of waking deficits (i.e., cumulative functions), which can reach levels of serious impairment within as little as 7-10 days (see preliminary results presented in Project 2). Sleep limited to less than 5 hr per night for 1-8 days results in decreased cognitive performance (Webb and Agnew, Jr. 1965; Wilkinson 1969; Frazier et al. 1971; Hamilton *et al.* 1972; Taub and Berger 1973; Herscovitch *et al.* 1980; Herscovitch and Broughton 1981; Tilley and Wilkinson 1984; Blagrove *et al.* 1995; Gillberg and Åkerstedt 1994; Dinges *et al.* 1997); reduced multiple sleep latency test (MSLT) values (Carskadon and Dement 1981; Carskadon and Dement 1982; Rosenthal *et al.* 1993; Gillberg and Åkerstedt 1994); increased subjective sleepiness (Taub and Berger 1973; Carskadon and Dement 1981; Herscovitch and Broughton 1981; Gillberg and Åkerstedt 1994); increased behavioral, affective, and somatic complaints (Blagrove *et al.* 1995); and polysomnographic evidence of increasing sleep pressure (Webb and Agnew, Jr. 1965; Taub and Berger 1973; Tilley and Wilkinson 1984; Rosenthal *et al.* 1993; Gillberg and Åkerstedt 1994).

Recent experiments performed on the waking neurobehavioral and physiological effects of sustained restriction of sleep for 10 days, reveals that performance and mood deficits accumulate reliably when sleep is restricted to either 4 hr or 6 hr per night, relative to 8 hours/night.

In addition to reduced sleep duration, the frequent reliance on hypnotic medications in space flight attest to the disrupted quality of sleep. For example, 19% of the space shuttle crew members on single shift missions and 50% of crew members in dual shift operations resorted to sleeping pill usage during the missions. Such a remarkably high incidence of hypnotic usage among the astronauts [3.3 and 8.7 times greater, respectively, than the percentage of Americans estimated to use hypnotics at any time in a given year (National Academy of Sciences, 1979)] indicates that on average the sleep of astronauts on the space shuttle is profoundly disturbed. In fact, a recent comprehensive review of drug log information collected on 89 U.S. space shuttle missions has revealed that sleeping pills account for approximately 80 percent of all medications taken by astronauts during space flight (L. Putcha, Ph.D., NASA Johnson Space Center, personal communication). In 1993, sleep was polygraphically recorded from an astronaut for four nights during a short space mission by Gundel *et al.* (Gundel *et al.* 1993). The results were consistent with prior subjective reports, with reduced sleep duration and increased number of awakenings, even though the first and last nights of the mission (usually the most disturbed) were not recorded for technical reasons.

Analysis of typical U.S. space shuttle astronauts' sleep-wake schedules reveals that misalignment of circadian rhythms may play a prominent role in these disturbances. First, space shuttle flights typically are launched at 6-8 a.m. EST (Santy and Davis 1990). Since crew members must begin their extensive pre-launch preparations six hours before launch, they must awaken between midnight and 2 a.m. on the day of launch. Thus, a 4-7 hour phase advance in their usual sleep-wake cycle--equivalent to that required for a trip from Washington, DC to Europe--must be achieved. Members of our team have therefore implemented a bright light treatment program for crew members of NASA's Space Shuttle (beginning with STS-35), which produced successful realignment of the endogenous circadian pacemaker driving the melatonin rhythm with the new sleep/wake cycle required for a dual-shift mission (Czeisler *et al.* 1991). This procedure is now routinely used on nearly all shuttle flights.

Since all crew activities in space are scheduled with respect to the moment of launch [that is, in Mission Elapsed Time (MET), rather than in local Houston or Orlando time], the shifted sleep schedule of crew members on the day of launch must typically maintained in space. Even though a new set of guidelines now limits the number of hours an astronaut's scheduled daily sleep episode can be shifted each day during a mission (Mission; *et al.* 1990), it is still not uncommon, due to operational constraints, for several consecutive sleep episodes during a mission to be scheduled on a 23-hour day rather than on the 24-hour day we maintain on earth. As laboratory studies have demonstrated, 23 hours can be outside the range of day lengths to which the human circadian timing system can adapt in response to a light-dark cycle consisting of ordinary room light during the daytime and darkness at night (Fookson *et al.* 1984; Wever 1979). In fact, this type of schedule is one of the most effective laboratory means of inducing sleep onset insomnia among normal subjects yet to be developed (Strogatz *et al.* 1987; Fookson *et al.* 1984). That may be why the Soviet space program has reportedly found that crew members' long-term adaptation to space flight is improved when the cosmonauts' sleep-wake schedule is maintained on Moscow time (Garshnek 1989). However, sleep in microgravity is disturbed even when great effort is made to avoid circadian rhythm disruption in the scheduling of sleep, as was recently done in a study conducted by Monk *et al.* (1998). They polygraphically recorded sleep and measured circadian rhythms of four male astronauts aboard a space shuttle (STS-78) orbiting Earth for 17 days and found that the astronauts obtained a decreased amount of sleep during flight (mean sleep duration = 6.1 h). Furthermore, they reported that all four astronauts showed a decrease in delta sleep, even though the schedule of this mission was specifically designed to avoid circadian rhythm disruptions.

Unfortunately, except under such experimental conditions, the NASA space shuttle's launch and landing constraints ordinarily preclude such synchronization of the astronauts' schedules with Houston or Orlando time (Santy and Davis 1990). It is thus not surprising that the astronauts are often forced to resort to sleeping pill use in order to obtain the sleep they need during their scheduled sleep episodes (Santy *et al.* 1988). Furthermore, shuttle

astronauts are typically scheduled for work duties and other waking activities for more than 12-hours per day, for 10-14 days in a row, without the opportunity for regularly scheduled days off to recover from cumulative sleep deprivation. Thus, while sleep is certainly possible in space, it is often fragmented or disturbed.

This experience has been shared by Russian cosmonauts. In fact, during an extended duration orbit, persistent insomnia led one cosmonaut to excessive hypnotic use, which was thought to have induced psychiatric symptoms (Graeber 1988). Cosmonauts on long-term missions appear to have been particularly vulnerable to the effects of fatigue, leading ground control to allow cosmonauts to sleep up to twelve hours per day by the end of very long missions (Graeber 1988). Greater attention must be paid to the development of countermeasures to avoid the deleterious effects of both acute and chronic sleep loss on the alertness and performance of crew members, particularly in anticipation of the long-duration missions planned aboard the International Space Station.

Execution of Protocols Without PI

Execution of scientific experiments in the remote and inaccessible space location is often compromised by the absence of highly trained experts such as PIs and by limited communication between spacecraft and earth. The application of artificial intelligence and expert systems is a promising tool in addressing the difficulties associated with performing experiments in space. Expert systems, in which human logic is modeled using expert systems "shell" such as LES (Learning Expert System) or CLIPS (C Language Integrated Production System), which provide a framework for rule-based programming, are currently being used routinely in many ground-based applications, particularly in the field of medicine (Shortliffe 1991). Areas include, acute care systems, decision support systems, educational systems, laboratory systems, quality assurance and administration systems and medical imaging systems. Although expert systems are not widely used in space applications, procedures and experiments carried out by astronauts in the remote space environment could potentially benefit from the implementation of expert systems as an intelligent decision aid.

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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Progress Report

PROJECT TITLE: PROJECT 1: CIRCADIAN ENTRAINMENT, SLEEP-WAKE REGULATION AND NEUROBEHAVIORAL PERFORMANCE DURING EXTENDED DURATION SPACE FLIGHT

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PERIOD OF PERFORMANCE: October 1, 1997 – September 30, 2000

EXECUTIVE SUMMARY

Long-duration exploration class space flight requires crew members to maintain a high level of cognitive performance and vigilance while operating and monitoring sophisticated instrumentation. However, the reduction in the strength of environmental synchronizers in the space environment leads to misalignment of circadian phase among crew members, coupled with restricted time available to sleep, results in sleep deprivation and consequent deterioration of neurobehavioral function.

Crew members are provided, and presently use, long-acting benzodiazepine hypnotics on board the current, relatively brief space shuttle missions to counteract such sleep disruption, a situation that is only likely to worsen during extended duration missions. Given the known carry-over effects of such compounds on daytime performance, together with the reduction in emergency readiness associated with their use at night, NASA has recognized the need to develop effective but safe countermeasures to allow crew members to obtain an adequate amount of sleep. Over the past nine years, we have successfully implemented a new technology for shuttle crew members involving bright light exposure during the pre-launch period to facilitate adaptation of the circadian timing system to the inversions of the sleep-wake schedule often required during dual shift missions (1). However for long duration space station missions it will be necessary to develop effective and attainable countermeasures that can be used chronically to optimize circadian entrainment.

Our current research effort is to study the effects of light-dark cycles with reduced *zeitgeber* strength, such as are anticipated during exploration class space missions, on the entrainment of the endogenous circadian timing system and to study the effects of a countermeasure that consists of scheduled brief exposures to bright light on the human circadian timing system. The studies are designed to address the following Specific Aims:

1) test the hypothesis that synchronization of the human circadian pacemaker will be disturbed in men and women by the reduction in LD cycle strength.

2) test the hypothesis that this disturbed circadian synchronization will result in the secretion of the sleep-promoting hormone melatonin during the waking day, disturbed sleep, reduced growth hormone secretion, and impaired performance and daytime alertness;

Results suggest that a strictly scheduled wake-sleep cycle with dim light levels, similar to that which astronauts are currently exposed on space shuttle missions, is sufficient to maintain entrainment of the human circadian pacemaker to the 24.0 hr day for most but not all subjects tested. No subjects entrained to a longer-than-24-hr day under similar conditions. Circadian misalignment resulted in disturbed sleep, impaired alertness and performance, secretion of melatonin during the waking day and reduced nocturnal growth hormone secretion. Preliminary studies suggest that stronger synchronizers, such as brighter light, will be necessary to entrain the longer-than-24-hour intrinsic circadian period (2) of all humans to the 24.0 day and other day lengths such as the ~24.65 solar day of Mars.

The results of the current research may have important implications for the treatment of circadian rhythm sleep disorders, such as delayed sleep phase syndrome and shift-work dyssomnia, which are anticipated to have a high incidence and prevalence during extended duration space flight such as planned for the International Space Station and astronaut missions to Mars.

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I. PROJECT RESEARCH ACTIVITY

Sustaining high levels of performance throughout extended duration space missions requires: 1) circadian entrainment of the intrinsic longer-than-24-hour period of the human circadian pacemaker (2); and 2) maintenance of an appropriate phase relation of the human circadian pacemaker to the sleep-wake schedule. In space, astronauts are often exposed to light-dark cycles that are characterized by either an abnormal period, i.e., 1.5 hours during earth orbit and 24.6 hours during Mars orbit, or by a reduced intensity, i.e., between 10-50 lux in the angle of gaze, when the space craft is illuminated artificially and under power constraints. Such light-dark cycles may be inadequate to maintain the appropriate phase-relation between the sleep-wake cycle and the circadian system. Such circadian phase misalignment can result in sleep disturbances, reduced attention, gastrointestinal disorders and impaired daytime alertness.

The current research was designed to evaluate: (1) whether entrainment of the human circadian pacemaker will be disturbed when the strength of the environmental light-dark cycle is reduced; and 2) whether abnormal entrainment to either the 24.0-hour Earth day or the 24.6 hour Mars day will result in disturbed sleep, impaired daytime performance, reduced growth hormone secretion during sleep and inappropriate secretion of the sleep-promoting hormone melatonin during the waking day. To test these hypotheses, we conducted 18 long-term 55-day inpatient trials utilizing methodologies that allow direct measurement of endogenous circadian phase and amplitude before, during and after extended duration exposure to an environment with a reduction in the strength of external synchronizing cues [i.e., low light intensities].

We have demonstrated that a scheduled dim light-dark rest-activity cycle, with a dim ambient light intensity similar to that used aboard the space shuttle middeck, is able to entrain most, but not all human subjects to a scheduled 24-hr day, whereas none of the human subjects scheduled to a 24.6-h day (the period of the axial rotation of Mars) were entrained to this weak synchronizer. Circadian phase misalignment to the 24.6-h day resulted in sleep disturbance (reduced sleep efficiency), endocrine disturbance (secretion of the sleep-promoting hormone melatonin during the waking day, reduced nocturnal growth hormone secretion and reduced cortisol levels), and impaired daytime alertness and neurobehavioral performance (reduced vigilance and cognitive performance). The degree of circadian misalignment to the 24.6-h day was found to be strongly dependent upon the period of each subject's circadian pacemaker, such that subjects with periods shorter than 24.0 hr demonstrated the greatest degree of circadian misalignment to the 24.6-h day. Increasing the ambient light exposure to 120 lux maximum allowed some but not all subjects to entrain to the 24.6 hr day. Due to concerns over high radiation exposure during the voyage to Mars and while on the planet's surface, NASA engineers have indicated that neither the spacecraft nor the Martian habitat may have windows. Our data suggest that most if not all astronauts would exhibit circadian misalignment if the space flight lighting conditions of ~10 lux on the windowless middeck of the space shuttle were present on the space craft while en route to Mars or on the Mars station during their ~540 day stay on Mars. Furthermore, simulations using the Kronauer mathematical model for the effects of light on the human circadian pacemaker suggest while normal room light (light ~100-150 lux in the angle of gaze (equivalent to ~200-300 lux ambient) is sufficient to entrain the human circadian pacemaker to the 24.0-h day with a normal phase angle, this intensity of light is insufficient to entrain all astronauts to the 24.65-h solar day of Mars. Both the model and current data reveal that those astronauts who will have the greatest difficulty adapting to the 24.65 h Martian day will be those with an endogenous circadian pacemaker that has a period shorter than 24 h, which we estimate represents ~25% of the population.

The data collected through support from the NSBRI demonstrate the negative consequences of circadian misalignment that can be expected during exploration class space missions given current conditions of space flight. These results also highlight the need to develop effective and attainable countermeasures to prevent circadian misalignment during an exploration class mission to Mars.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

During the past 3 years of NSBRI grant support we have conducted "Basic Research and Research To Prove Feasibility" (Levels 1-4), as defined in the NSBRI 00-01 research announcement of February 22, 2000, in order to understand the fundamental mechanisms of circadian entrainment in humans and the problem of circadian entrainment of humans to the ~24.65-h solar day of Mars. Other preliminary research supported by NASA shows that intermittent light exposure is effective in resetting the human circadian pacemaker (3;6), however, we have yet to determine its efficacy to entrain humans to the 24.65-h day. We have proposed additional research studies to addresses several themes outlined in the NSBRI program announcement for the Human Performance, Sleep and Chronobiology Team to develop countermeasures to promote circadian alignment, sleep and performance during exploration class space missions. In addition, the maintenance of circadian entrainment, sleep and normal endocrine function by the proposed intermittent bright light countermeasure have implication for other NSBRI teams such as the bone, muscle and immune function teams. The countermeasure readiness of intermittent light exposure is in the "Countermeasure Development" stage (Levels 4-5). Results from proposed studies may allow this countermeasure to move from Levels 4 and 5 to Levels 6 and 7 and achieve "Countermeasure Demonstration" preparing it for validation with human subjects in space flight.

Development of optimal countermeasures for adaptation of astronauts to non-24-hour-day lengths during exploration class space missions requires a systematic research effort to correct key deficiencies in our present state of knowledge, which include:

1. *Effects of entrainment to longer-than-24-hour sleep-wake schedules on: sleep structure, sleep consolidation, daytime alertness and performance.* While sleep maintenance insomnia and daytime fatigue occur as a result of misalignment between the circadian pacemaker and sleep-wake schedule, sleep structure, sleep consolidation, daytime alertness and performance has seldom been quantified experimentally during entrainment to a longer-than-24-hour day.
2. *Effectiveness of brief exposures to bright light in normalizing: circadian entrainment, sleep consolidation, daytime alertness and cognitive performance.* Our recent preliminary data indicate that intermittent exposure to bright light can have a significant resetting effect on the human circadian pacemaker. If timed appropriately (i.e., shortly before bedtime), brief exposures to bright light should be able to effectively correct an adverse circadian phase of entrainment.
3. *Predictive mathematical models for the effects of non-24-hour schedules on circadian phase and amplitude.* Kronauer's mathematical model on the effect of light on the human circadian pacemaker has been an important research and application tool (4). In fact, Kronauer's biomathematical model has served as the cornerstone for designing the bright light exposure patterns used to treat NASA shuttle crewmembers during the pre-launch period (1). However, due to the paucity of data on the effects of entrainment to non-24-hour day lengths, the conditions anticipated during exploration class space missions, the mathematical model's predictions cannot be validated until further research studies have been conducted.

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III. APPENDICES:

A. Project Research Data

Hypotheses and Specific Aims from Original Proposal and Results

Sustaining high levels of performance throughout extended duration space missions requires: 1) circadian entrainment of the intrinsic longer-than-24-hour period of the human circadian pacemaker to the 24-hour day; and 2) maintenance of an appropriate phase relation of the human circadian pacemaker to the 24-h sleep-wake schedule. In space, astronauts are often exposed to light-dark cycles that are characterized by either an abnormal period, i.e., 1.5 hours during earth orbit, or by a reduced light intensity, i.e., between 10-50 lux in the angle of gaze, when the space craft is illuminated artificially and under power constraints. However, there is a critical deficiency in knowledge regarding the minimum intensity of light required to maintain the appropriate phase-relation between the 24-h sleep-wake cycle and the circadian system and thereby avoid misalignment of circadian phase. It is essential to correct this knowledge gap, since such circadian phase misalignment can result in sleep disturbances, reduced attention, gastrointestinal disorders and impaired daytime alertness.

Based on these preliminary results, three testable hypotheses were proposed, two of which were evaluated. Testing these hypotheses was critical for the development of effective countermeasures to prevent circadian misalignment: 1) that synchronization of the human circadian pacemaker will be disturbed in men and women by the reduction in LD cycle strength during the simulated lighting conditions of space flight; 2) that synchronization of the human circadian pacemaker to the 24.6 hr day of Mars will be disturbed in men and women; 3) that this disturbed circadian synchronization will result in the secretion of the sleep-promoting hormone melatonin during the waking day, disturbed sleep, reduced growth hormone secretion, and impaired performance and daytime alertness; 4) that three brief daily exposures to evening bright light (10,000 lux) will reestablish normal entrained circadian phase, resulting in improved sleep consolidation, normalized sleep structure and endogenous growth hormone secretion and enhanced daytime performance on the 24.6 hr Martian day.

To test hypotheses 1 thru 3, a long-term (55-day) clinical trial was conducted utilizing methodologies that allowed direct measurement of endogenous circadian phase and amplitude before, during and after extended duration exposure to an environment with a reduction in the strength of external synchronizing cues [i.e., low light intensities]. Testing of hypothesis 4 was delayed after guidance and consultation from the External Advisory Committee and the Board of Scientific Counselors.

In Year 1-3, experiments were conducted to test Hypotheses 1, 2 and 3. We anticipated that when subjects were maintained in dim room light conditions of 8 or 120 lux maximum and scheduled to multiple days, the endogenous circadian pacemaker would oscillate with a period that was different (24.1 to 24.2 hrs) from the period of the sleep-wake/work cycle (24.0 or 24.6 hrs). We expected that a LD 16:8 cycle of 8 or 120 lux light vs. darkness to be sufficient to maintain circadian entrainment to the 24 hr day, but at an adversely delayed phase. In subjects maintained on the 24.6 hr Mars day, we expected the imposed LD cycle to be too weak to maintain entrainment.

Blood was sampled for melatonin during three 58 hr sampling windows throughout the intervening study days between the first and second phase estimations (constant routines) of the protocol (see below). These sampling intervals allowed us to characterize the time course of circadian entrainment for the endogenous melatonin and cortisol rhythms.

Twelve 28-hour forced desynchrony days throughout the intervening experimental days between the second and third phase estimations (constant routines) of the protocol allowed us to quantify the intrinsic period of the endogenous circadian temperature and melatonin rhythms.

This allowed us to determine the extent to which the subjects' entrained circadian phases were dependent upon the underlying period of their circadian pacemakers.

Specific Research Protocol

Experimental Subjects. Eighteen healthy male and female volunteer subjects (age 31.9 ± 6.15, range 20-41) were studied. All subjects were required to pass a rigorous health screening and also maintain a regular sleep/wake schedule for three weeks prior to the start of study.

Experimental Procedures. The protocol was divided into 8 segments:

Segment A1 (Ambulatory Baseline) consisted of a minimum of 7 days, during which wrist activity, and light levels were recorded using ambulatory-recording devices while the subjects are at home on a normal routine, maintaining a sleep log.

Segment A2 (Adaptation) began with admission to the Intensive Physiologic Monitoring (IPM) Unit of the Brigham & Women's Hospital on Experimental Day 1 and ended on the morning of Experimental Day 7. Physiologic and neurobehavioral monitoring commenced upon admission on Experimental Day 1 and continued throughout the duration of the study. Each day, subjects were required to perform a battery of neurobehavioral tests every two waking hours. Subjects continued to sleep and wake at their regularly scheduled times for six normal scheduled nights. Subject's sleep was recorded on all experimental nights. Blood was sampled for melatonin once per hour, cortisol every thirty minutes starting from Experimental Day 5 until the end of Segment A3 below. Human growth hormone was sample for 36 h on Experimental Days 5-6.

Segment A3. (Endogenous Circadian Phase Assessment-ECPA) began on the morning of Experimental Day 7 and ended on the evening of Experimental Day 8. Upon waking on Experimental Day 7, the subjects were placed on a constant routine protocol for 40 hours after which time subjects underwent an 8-hour recovery sleep. The constant routine protocol, as is true with the rest of the protocol from this point forward, was conducted in dim room light. Blood was being sampled for melatonin every sixty minutes and cortisol every 30 minutes until the morning of Experimental Day 9.

Segment A4. 25 successive 24- or 24.6- h days began on the morning of Experimental Day 9. Each day consisted of 16-hours of wakefulness and 8-hours of sleep. Light exposure was being maintained at < 8 or < 120 lux maximum during all waking hours (Table 1). In addition to the blood sampled during the constant routines, three 58-hour "windows" of intensive physiological monitoring took place during Experimental Days 13-15, 20-22, and 27-29. During these windows, blood was sampled for melatonin and for cortisol every thirty minutes. Blood was also sampled for human growth hormone on days 27-29. Polysomnographic sleep recordings occurred on all nights.

Segment A5 (ECPA Reassessment) consisted of a repetition of the constant routine and recovery sleep that was used in the protocol for subjects in Segment A3.

Segment A6 (Forced Desynchrony) consisted of 12 consecutive 28-hour days (fourteen 24-hour days) beginning on the morning of Experimental Day 36. Each day consisted of 18.33 hours of wakefulness and 9.67 hours sleep. Subjects were maintained in either < 8 or < 15 lux of light during all waking hours. This segment continued until the morning of experimental day 50. Blood was sampled for melatonin every hour during this segment of the protocol. Polygraphic wake and sleep recordings occurred throughout this segment of the protocol.

Segment A7. (ECPA Reassessment) consisted of a repetition of the constant routine and recovery sleep that was used in the protocol for subjects in Segments A3 and A5. The constant routine began on Experimental Day 50 and continued for 40 hours until the evening of Experimental Day 51. The segment ended on the morning of Experimental Day 52.

Segment A8. (Recovery) consisted of two or three standard days and three nights of recovery sleep. Subjects were readjusted to normal lighting (150-500 lux) and sleep times and then discharged from the study.

Table 1. Summary of lighting conditions in the experimental research suites: Relationship between the maximum, ambient and angle of gaze measurements of light intensity.

Lighting Intensity

Maximum	Ambient Lighting	Angle of Gaze
< 8 lux	< 3 lux	~1.5 lux
< 15 lux	< 5 lux	~3 lux
< 120 lux	< 50 lux	~25 lux
< 1500 lux	< 1100 lux	~450 lux

The < 120 lux maximum light intensity is equal to a dimly lit room whereas the < 8 lux maximum is very dim and is similar to the light intensity that was recently recorded on the mid-deck of the space shuttle on STS-90.

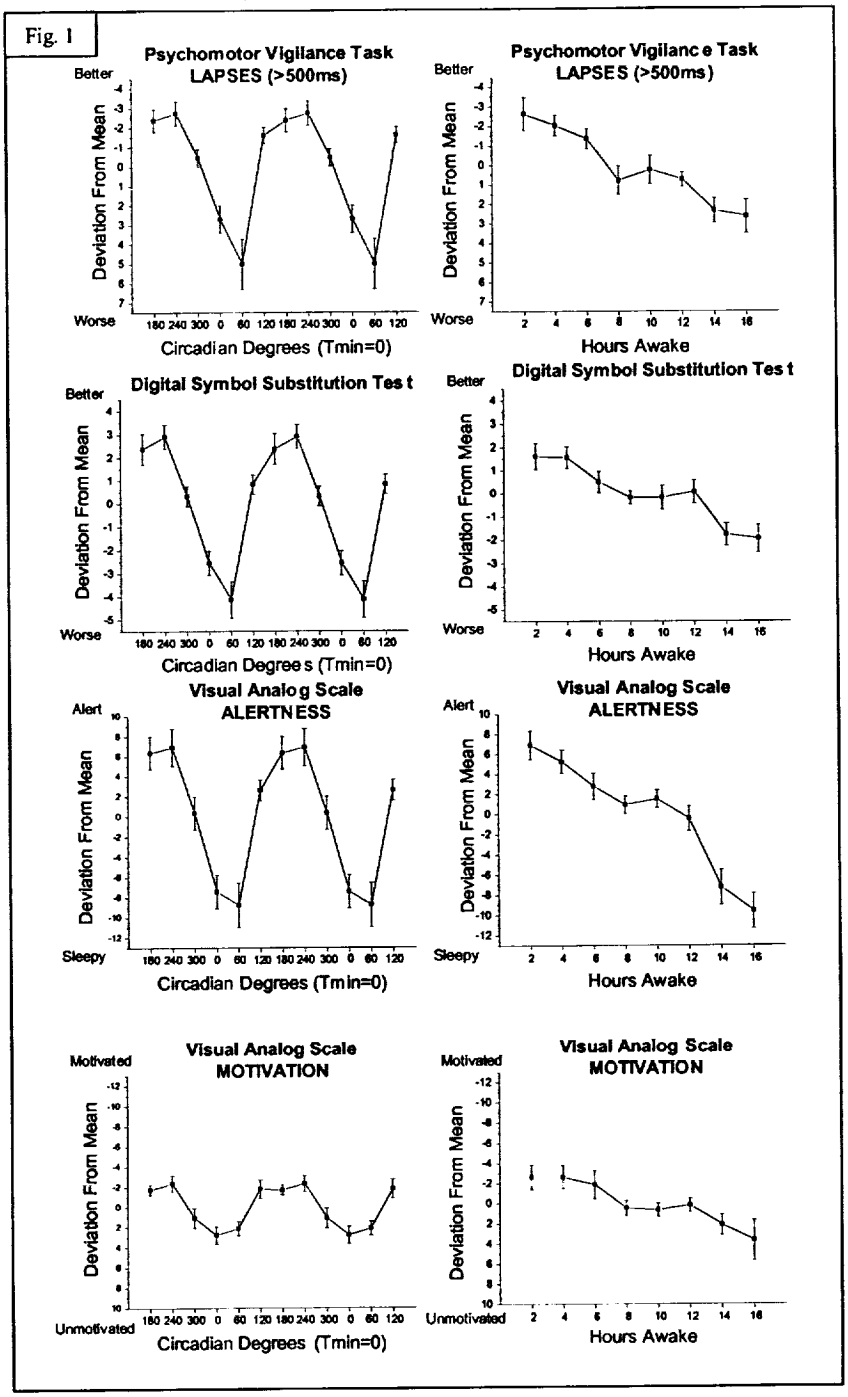
Table 2. Number of subjects in each experimental condition

Lighting Intensity

	< 120 Lux max	< 8 Lux max
Light-Dark Cycle 24 hr light-dark cycle of Earth	2 completed	6 completed
24.6 h Axial Rotation of Mars	4 completed	6 completed

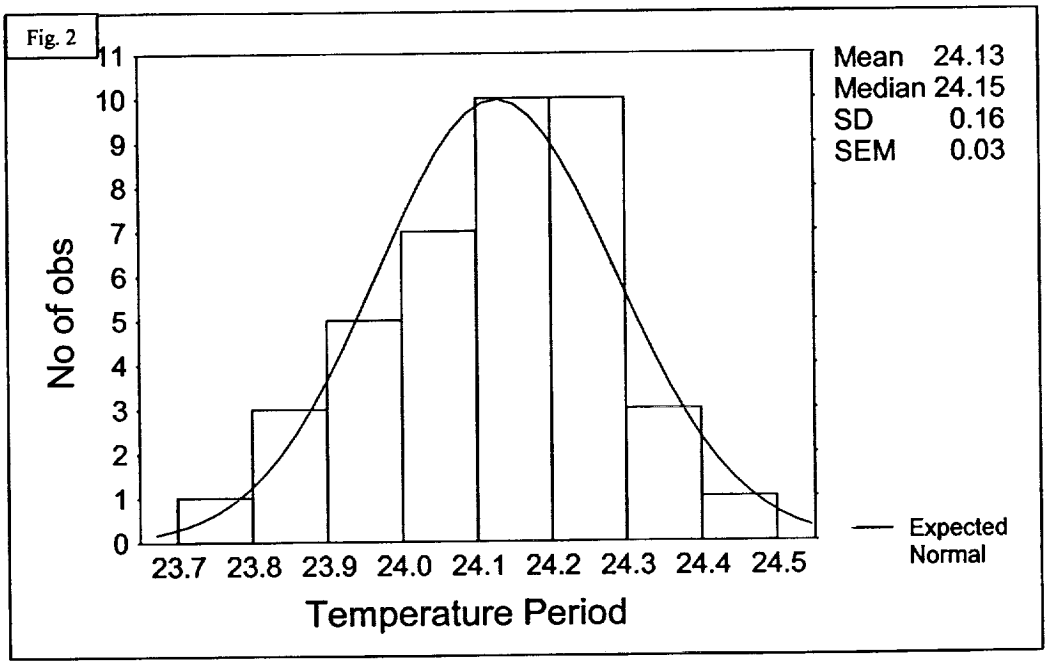
Circadian and Homeostatic Regulation of Neurobehavioral Performance Alertness and performance are regulated by the output of the endogenous circadian pacemaker and a wake-dependent homeostatic process. Data from 15 subjects who completed the two weeks of forced desynchrony (28-h days), demonstrate results consistent with our previous findings showing the lowest alertness and worst performance (vigilance and cognitive throughout) to occur just after the temperature minimum and near the end of the waking day (Figure 1). Significant circadian and time awake effects were observed for all measures ($P<0.05$). This is consistent with data that Cajochen et al. collected in another NSBRI supported project demonstrate that the occurrence of slow eye movements and involuntary microsleep episodes just after the endogenous temperature nadir. The data also demonstrate that circadian and homeostatic processes regulate motivation to perform in a similar manner. These findings show that as little of a 2-4 hr change in phase angle between the circadian pacemaker and sleep-wake schedule can have severe consequences for the maintenance of alertness and performance across the waking day.

Fig. 1



Evaluation of Intrinsic Circadian Period in Human To date, we have studied 40 healthy young men and women, ages 18-41 years, to evaluate the range of circadian periods in humans (15 of these subjects are from the current NSBRI study). We imposed a period of either 20- or 28-h days that included scheduled sleep for one-third of the 'day' and wake for the remaining two-thirds. Ambient light levels were kept at < 15 lux throughout. Core body temperature was measured and endogenous circadian period was estimated by non-orthogonal spectral analysis (NOSA) to reveal the best fitting sought-for period, having taken into account the imposed period of the sleep-wake cycle (2). The average (± SD) estimated intrinsic temperature period for the young subjects was 24.13 ± 0.16 (range 23.77 - 24.48 h). Approximately 25% of subjects exhibit circadian temperature periods equal to or shorter than 24.0-h (Figure 3). Assuming a normal distribution of periods, ± 3 SD should encompass 99% of the population with a hypothesized range of 23.65 to 24.61-h. These data reveal that the average human would require a 0.52-h daily phase delay to adjust to the ~24.65-h solar day of Mars. This adjustment is 4 times greater than the daily adjustment required by the same human to entrain to the 24.0-h day. Furthermore, ~25% of astronauts would require a 0.65-h or greater daily phase delay to entrain to Mars and the humans with the shortest circadian periods require an ~1.0-h daily adjustment. Success of the planned exploration class mission to Mars would be severely compromised even if one crewmember were to fail to adapt their circadian system to the Mars day. Therefore, any countermeasure used to entrain the human circadian pacemaker to the 24.65-h day must be able to produce a daily phase adjustment of at least 1-h to ensure proper circadian alignment for all crewmembers.

Fig. 2



Near-24-Hour Entrainment Limits of the Human Circadian Pacemaker The primary goal of our current NSBRI grant was to evaluate entrainment to environmental synchronizers of weak entraining strength using dim light-dark cycles similar to that aboard the windowless mid-deck of the space shuttle as we measured aboard STS-90. We studied 12 healthy subjects for up to 55 days each in the laboratory to assess the entrainment limits of the human circadian pacemaker to dim light-dark rest-activity schedules. In 15 subjects, lighting conditions were as follows: < 8

lux maximum at 72" in the direction of the light fixture, ~1.5 lux in the angle of gaze; whereas for 6 subjects, lighting conditions were: < 120 lux maximum at 72" in the direction of the light fixtures, ~25 lux in the angle of gaze. Subjects were scheduled to either a 24.0 or 24.6-h day. Seven of eight subjects studied on the 24.0-h day maintained normal phase relationships between the timing of their melatonin and core body temperature rhythms and the timing of the scheduled sleep-wake cycle (Figure 3a). All but one subject achieved the daily phase adjustment required by their pacemaker to entrain to the 24.0-h day. Figures 4a and 4b show representative examples of melatonin levels above the 24-h mean (horizontal lines), during scheduled sleep episodes (open boxes). Figure 4c shows the results for the one subject who failed to entrain to the 24.0-h day. The forced desynchrony protocol revealed this subject to have the intrinsic period furthest from 24.0-h (24.36 ± 0.01 h). Subjects scheduled to 24.6-h days in dim light (Figure 4b) were unable to achieve the daily phase shift required to entrain to the non-24.0-h schedule in dim light. Instead, all humans tested on the 24.6-h day in ~1.5 lux showed abnormally advanced phase angle between the timing of the melatonin rhythm and the timing of the scheduled sleep episode on the 24.6-h day. Figures 5a and 5b show plasma melatonin data for the subject with the circadian period furthest and closest to the 24.6-h day, respectively. Melatonin levels during 24.0-h baseline conditions were high during the scheduled sleep episode. However, during the entrainment protocol, the scheduled 24.6 h day in ~ 1.5 lux was insufficient to synchronize their circadian pacemaker, resulting in high levels of melatonin during the waking day and low levels at night. The one subject who clearly did not entrain to the 24.6-h day in ~25 lux showed a change in phase angle such that their temperature minimum occurred 2-h prior to bedtime and with high melatonin levels during the waking day (Figure 4c). This subject required a phase delay of 45 min per day and achieved only 31 min per day. Three of four subjects tested on the 24.6-h day in ~25 lux maintained phase angles in the normal range, but one of these three did not appear to achieve the daily adjustment required by their pacemaker to entrain to the 24.6-h day as assessed by linear fits to melatonin onsets during forced desynchrony and the entrainment protocol respectively (required a daily 31 min phase delay; achieved 24 min per day). Thus, it is unclear whether or not this subject is entrained to the 24.6-h day with a normal or abnormal phase angle.

Fig. 3

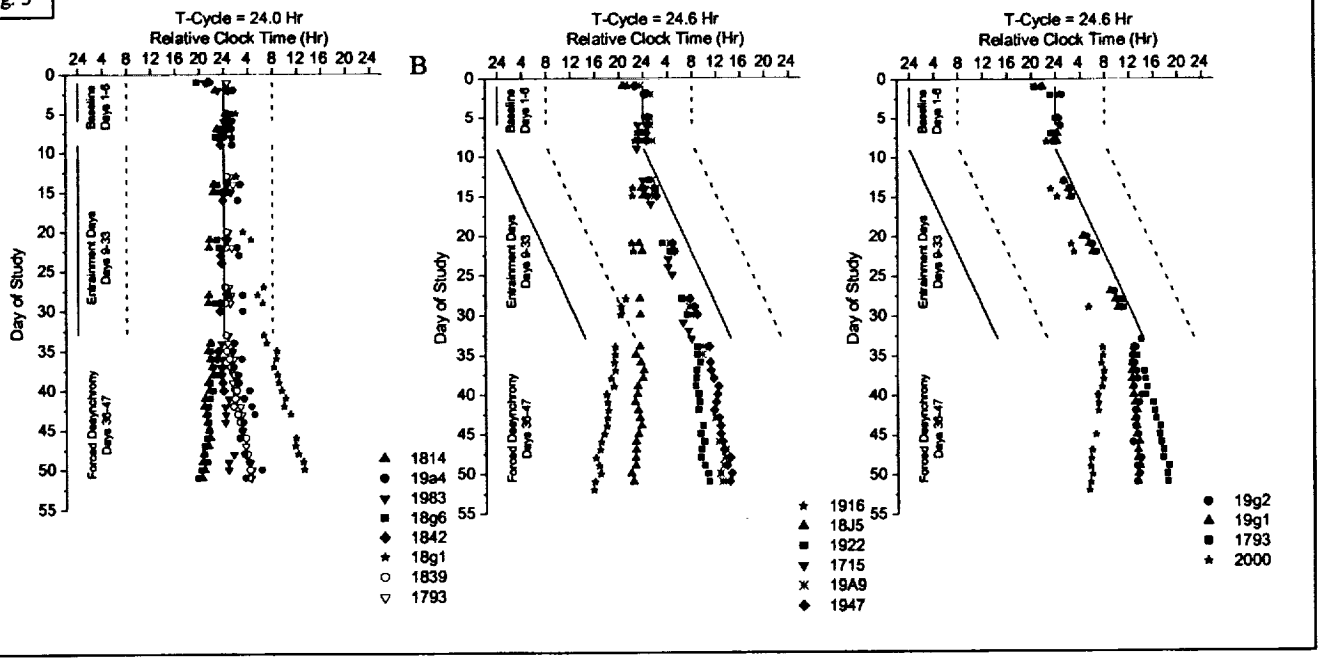


Fig. 4

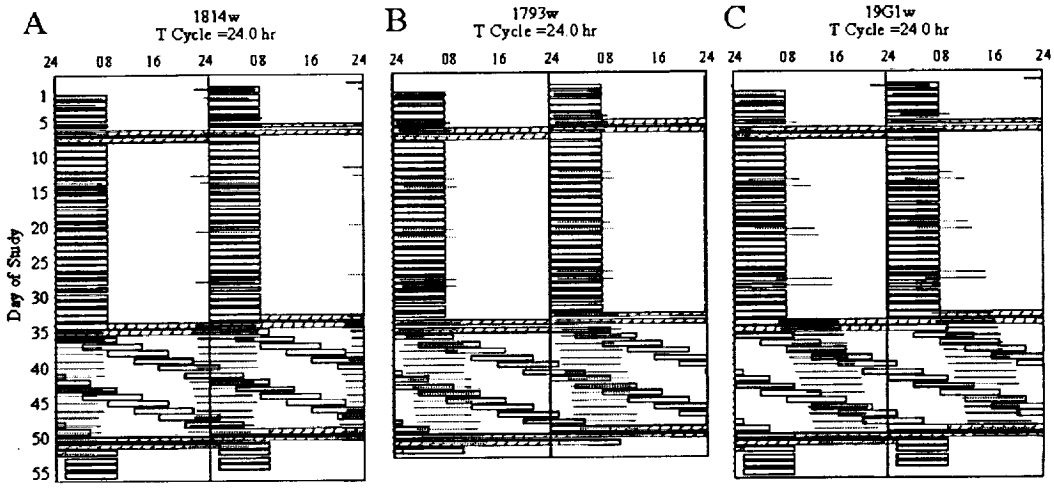
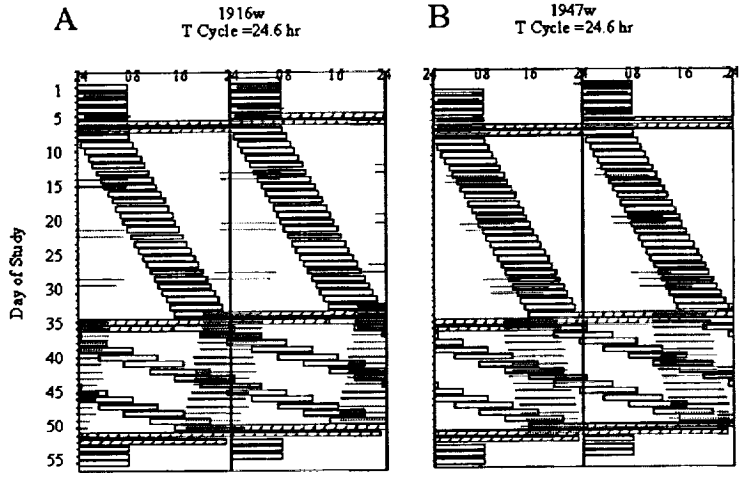
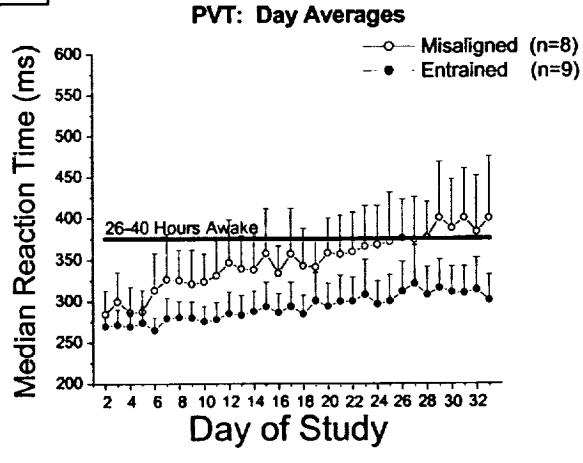


Fig. 5



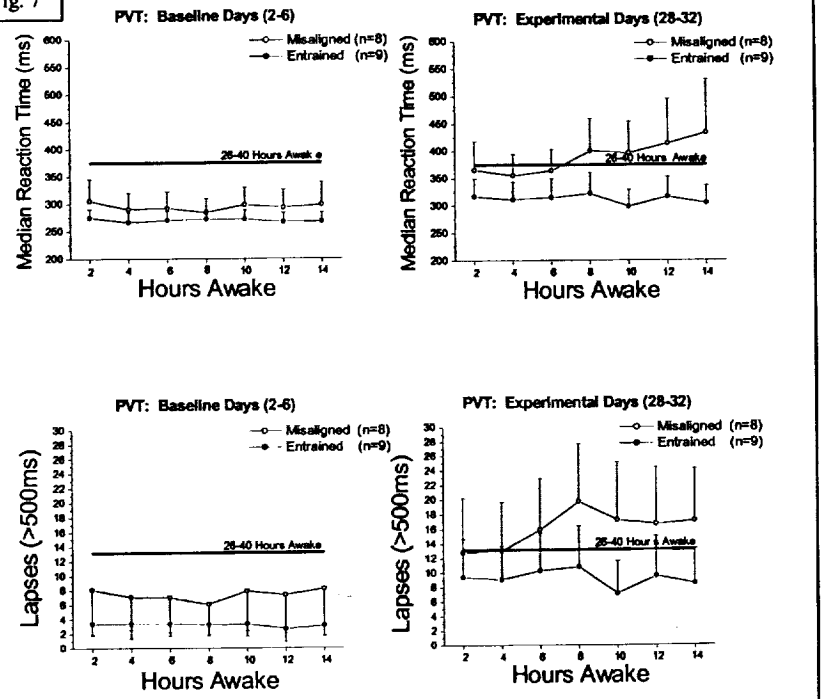
Impaired Neurobehavioral Performance During Circadian Misalignment We also examined neurobehavioral function during the entrainment studies to the 24.0 and 24.6-h days. Daily performance averages were computed for subjects with apparently normal phase angles (designated as entrained) and for those with abnormal phase angles (not-entrained). We assessed vigilance performance every 2-h during wakefulness using the Psychomotor Vigilance Task (PVT). This test has been shown to be sensitive to sleep loss, misalignment of circadian phase and countermeasures such as caffeine. Also, this test does not exhibit a learning curve unlike many other cognitive performance tests. The primary outcome measures analyzed were median reaction time and lapses (response times > 500 ms). Visual analog scales were used to assess subjective alertness. Results of these analyses show that vigilance performance worsened across the entrainment protocol

Fig. 6



to a greater degree in the subjects who were not entrained compared to those whose pacemakers were entrained (Figure 6). The bold line on each graph provides average performance for these same subjects after 24 hours of total sleep deprivation on constant routine 1. These data show that near the end of the entrainment protocol, subjects who failed to entrain exhibited performance levels on average that were equal to those seen after total sleep deprivation. Subjective alertness levels however did not differ among groups nor did they change across the entrainment period suggesting that subjects were not able to accurately assess their level of alertness. We also examined performance across the day and compared performance levels average across 5 baseline days to performance average across the last 5 24.0 or 24.6 h days. Figure 7 shows that performance was worse in the group that did not entrain, and that performance worsened across the day for this group. Higher reaction times and lapses equal worse performance. There were significant differences between entrained and misaligned subjects during days 28-32 with respect to the number of hours awake ($P<0.05$). Performance levels for subjects who maintained entrainment exhibited consistent levels of performance across the day. These results are consistent with what would be expected based upon an opponent process model of performance regulation by the circadian pacemaker and a wake-dependent process (sleep homeostasis). Subjects, whose pacemakers were entrained to the scheduled sleep-wake cycle exhibit the normal opponent processes such that circadian and homeostatic processes interact to maintain stable levels of performance across the waking day. The opponent processes for subjects who did not entrain were out of phase and resulted in worse performance later in the waking day.

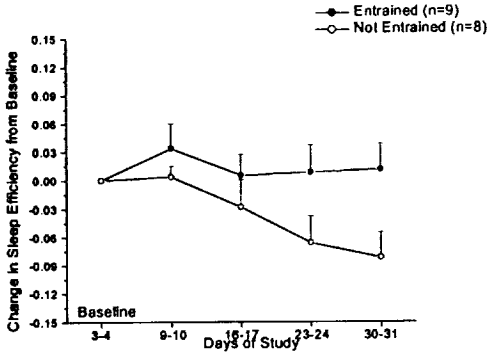
Fig. 7



Even though performance was better for subjects who were entrained, their performance also worsened across the protocol suggesting that dim light or minor changes in circadian phase may be detrimental to waking performance. Other possible explanations include that 8-h of scheduled sleep is insufficient to maintain optimal performance levels under schedules without a day off from work, or that specifics of the PVT test may lead to this pattern of results. Dinges and colleagues have observed a similar worsening in performance after 14 days of scheduled 8-h of sleep in normal room light (personnel communication).

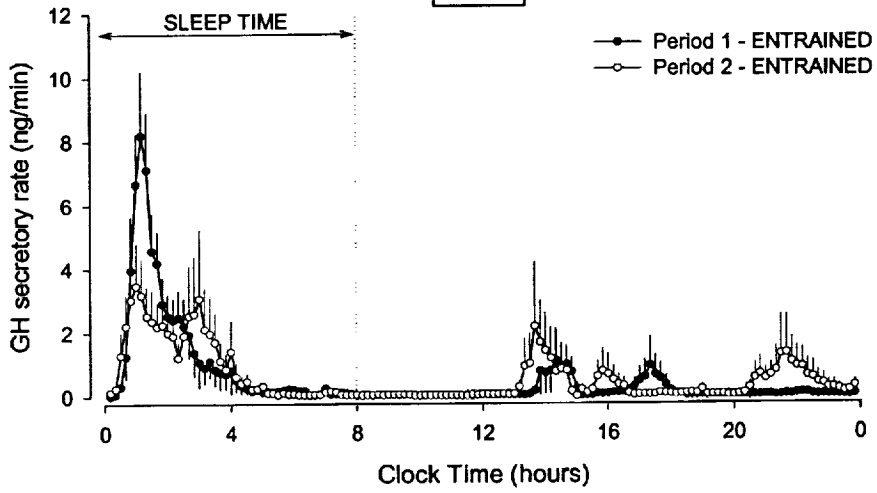
Impaired Sleep and Endocrine Function During Circadian Misalignment We also assessed sleep and endocrine function during entrainment to 24.0 and 24.6-h day lengths. Sleep recordings

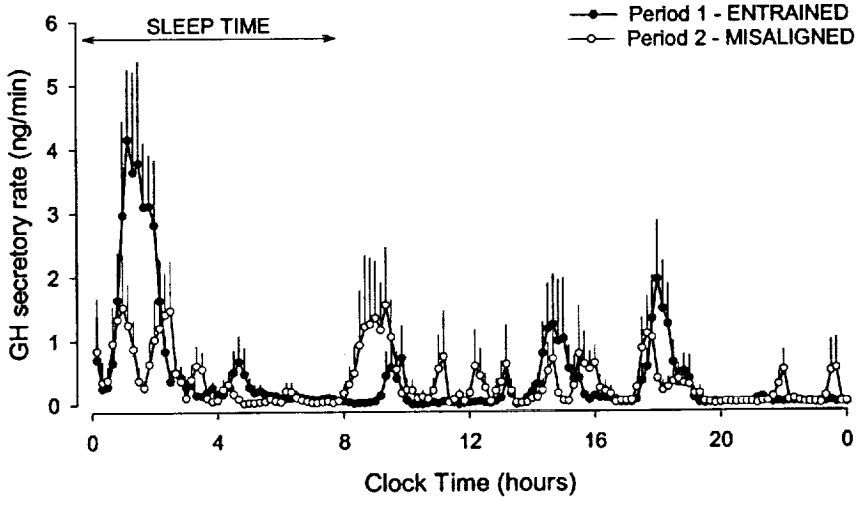
were scored according to standard criteria (5). Sleep data were averaged across two consecutive nights, during which blood was not sampled, every 5-6 days during the baseline and entrainment protocols. Sleep efficiency worsened across the protocol for subjects who failed to entrain to the sleep-wake cycle, whereas entrained subjects maintained a consistent pattern of sleep efficiency across the protocol. We should note that the impairment of sleep fluctuated daily in the not entrained group such that sleep efficiency would be poor on one night and improve on the next night, presumably due to homeostasis. Wake after sleep onset showed a similar results to sleep efficiency. Subjects who did not entrain also showed a significant increase in stage 3/4 sleep on days 30/31 suggesting increased homeostatic sleep pressure, whereas entrained subjects maintained similar levels of stage 3/4 sleep across the study. There were no differences in stage 2 or REM sleep between groups nor were there any changes across the entrainment protocol.



We also observed disruption of endocrine function associated with circadian misalignment and impaired sleep. Blood was sampled for human growth hormone every 10 minutes during two 36-h sampling windows during baseline (days 5-6) and near the end of the entrainment protocol (days 27-28). Analyses were restricted to 8-h of sleep for subjects on the 24.6-h day so that their data could be compared to their 8-h baseline sleep episodes. A deconvolution procedure was used to estimate the amount of growth hormone secreted. Subjects who did not entrain to the sleep-wake cycle showed significantly less growth hormone secretion

Fig. 9

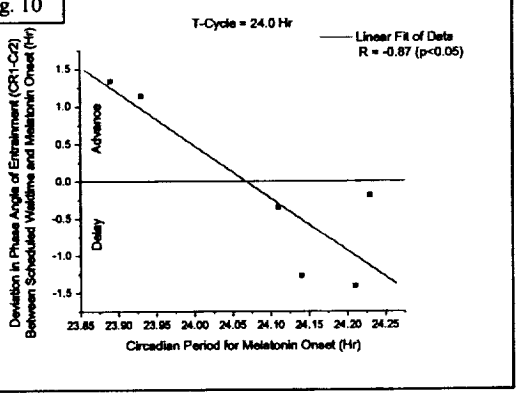


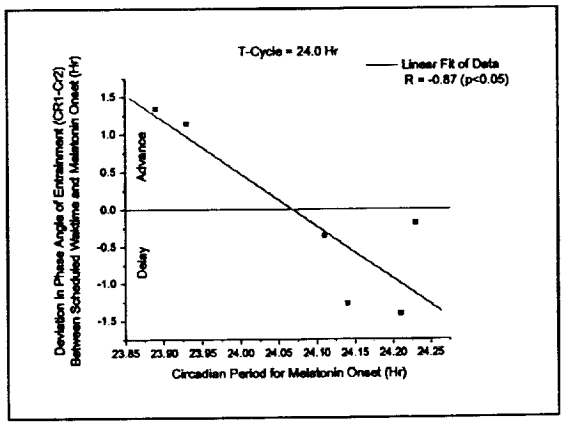


during the sleep period compared to their own level of secretion during baseline ($P<0.05$). Subjects who entrained also showed a decrease in growth hormone secretion, but this decrease was not significantly different from baseline ($P=0.18$). Cortisol levels were assessed every 30 minutes during the same time period. Twenty-four hour mean cortisol levels were significantly lower in subjects who did not entrain compared to the levels in the same individuals at baseline (7.39 ± 0.49 ug/dl versus 8.98 ± 0.57 ug/dl at baseline) ($P<0.01$). Subjects who entrained showed similar cortisol secretion during entrained and baseline days (7.58 ± 0.64 ug/dl versus 7.10 ± 0.41 ug/dl at baseline) ($P=0.42$). Taken together, the cortisol and growth hormone data suggest that circadian misalignment and associated sleep loss disturb normal endocrine function. The lower growth hormone secretion for subjects who did not entrain has implication for the maintenance of muscle and bone, which are already compromised by altered gravity during space flight. Lower cortisol levels may also have implications for numerous metabolic processes, immune function and responses to stress.

We also assessed the relationship between circadian period as assessed during forced desynchrony and the change in phase angle of entrainment to the 24.0-h day or the degree of circadian misalignment to the 24.6-h day. As can be seen in Figure 10 there is a significant relationship between the intrinsic period and the entrained phase ($r = -0.87$, $P<0.05$) such that the phase angle drifted earlier for subjects with shorter-than-24-hour periods and the phase angle for those with longer-than-24-hour periods drifted later. Data from subjects who did not entrain to the 24.6-h day show that the degree of circadian misalignment was also strongly related to period ($r=-.96$, $P<0.01$). All subjects scheduled to the 24.6-h day in dim light (~1.5 lux) exhibited an abnormal advanced phase and as expected, subjects who had circadian periods shorter-than-24-hour showed the greatest degree of circadian misalignment.

Fig. 10





List of publications supported through NSBRI funding and appropriately acknowledged.

Articles in journals (peer reviewed)

Czeisler, C.A., Duffy, J.F., Shanahan, T.L., Brown, E.M. Mitchell, J.F., Rimmer, D.W., Ronda, J.M., Silva, E.J., Allan, J.S., Emens, J.S., Dijk, D.J., and Kronauer, R.E. Stability, Precision and Near 24-Hour Period of the Human Circadian Pacemaker. Science. 1999; 284: 2177-2181.

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Czeisler, C.A, Klerman, E.B. Circadian and Sleep-dependent Regulation of Hormone Release in Humans. Recent Prog.Horm.Res. 1999; 54:97-132.

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National Space Biomedical Research Institute

FINAL PROJECT REPORT

PROJECT TITLE: **Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation during Space Flight**

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EXECUTIVE SUMMARY

This project is concerned with identifying ways to prevent neurobehavioral and physical deterioration due to inadequate sleep in astronauts during long-duration manned space flight. The performance capability of astronauts during extended-duration space flight depends heavily on achieving recovery through adequate sleep. Even with appropriate circadian alignment, sleep loss can erode fundamental elements of human performance capability including vigilance, cognitive speed and accuracy, working memory, reaction time, and physiological alertness. Adequate sleep is essential during manned space flight not only to ensure high levels of safe and effective human performance, but also as a basic regulatory biology critical to healthy human functioning.

There is now extensive objective evidence that astronaut sleep is frequently restricted in space flight to averages between 4 hr and 6.5 hr/day. Chronic sleep restriction during manned space flight can occur in response to endogenous disturbances of sleep (microgravity, motion sickness, stress, circadian rhythms), environmental disruptions of sleep (noise, temperature, light), and curtailment of sleep due to the work demands and other activities that accompany extended space flight operations. The mechanism through which this risk emerges is the development of cumulative homeostatic pressure for sleep across consecutive days of inadequate sleep. Research has shown that the physiological sleepiness and performance deficits engendered by sleep debt can progressively worsen (i.e., accumulate) over consecutive days of sleep restriction, and that sleep limited to levels commonly experienced by astronauts (i.e., 4 - 6hr per night) for as little as 1 week, can result in increased lapses of attention, degradation of response times, deficits in complex problem solving, reduced learning, mood disturbance, and disruption of essential neuroendocrine functions.

The prevention of cumulative performance deficits and neuroendocrine disruption from sleep restriction during extended duration space flight involves finding the most effective ways to obtain sleep in order to maintain the high-level cognitive and physical performance functions required for manned space flight. There is currently a critical deficiency in knowledge of the effects of how variations in sleep duration and timing relate to the most efficient return of performance per unit time invested in sleep during long-duration missions, and how the nature of sleep physiology changes as a function of sleep restriction and performance degradation. The primary aim of this project is to meet these critical deficiencies through utilization of a **response surface experimental paradigm**, testing in a dose-response manner, varying combinations of sleep duration and timing, for the purpose of establishing how to most effectively limit the cumulative adverse effects on human performance and physiology of chronic sleep restriction in space operations.

To develop a response surface models of the best sleep-wake schedules for astronauts, 90 healthy men and women underwent a 14-day ground-based laboratory protocol involving random assignment to one of 18 sleep-ration cells, each involving the same sleep ration for 10 consecutive days. The sleep-ration assignments involved four nocturnal anchor sleep durations (4.2, 5.2, 6.2, 8.2 hr) and six diurnal nap sleep durations (0.4, 0.8, 1.2, 1.6, 2.0, 2.4 hr) crossed to yield a total of four anchor-sleep-only conditions, and 14 anchor + nap sleep conditions, and spanning a dynamic range of cumulative sleep debts (i.e., from 0 to 40 hr in a 10-day period). Throughout the 14 days, subjects lived in conditions that simulated aspects of prolonged space flight (e.g., confined small groups, social and environmental isolation, controlled diet and activity) and underwent a wide range of quasi-continuous neurobehavioral performance tests and continuous physiological monitoring of brain activity, sleep physiology, core body temperature, and behavioral motility. The laboratory environment was designed to simulate the low light, tight quarters, and lack of social contact with the outside world that will characterize long-duration space flight.

Data acquisition in this project has been completed response surface model (RSM) development and hypothesis testing on the large set of neurobehavioral and physiological outcomes are currently underway. Thus far, the results of the experiment have revealed that subjects are able to achieve significant levels of physiological sleep when both anchor (nocturnal) and nap (diurnal) sleep opportunities are chronic (i.e., part of a daily schedule), regardless of the time in bed allowed for sleep. Even daytime nap opportunities as brief as 0.4 hr (24 minutes) consistently resulted in physiological sleep. This indicates that the diverse range of restricted anchor + nap sleep durations tested in this protocol will likely result in sleep if used by astronauts. Response surface models fit to neurobehavioral data reveal that the combination of a restricted duration anchor sleep and a diurnal nap can help prevent the development of cumulative deficits that can occur when only restricted anchor sleep is permitted (as is currently typical in space flight). Moreover, all response surface models being evaluated for optimizing performance, mood, sleep physiology, and hormonal profiles in the face of restricted total sleep time include the duration of nocturnal anchor sleep, the duration of diurnal nap sleep, age, gender, and baseline individual differences. Thus, the results of this study will permit us to estimate the relative contributions of astronaut demographics (age, gender) and individual abilities (baseline differences) to cumulative neurobehavioral deficits due to sleep restriction. This permits a more precise estimate of how a given sleep-wake schedule will likely affect different crews.

This experiment is the first ground-based study to utilize the slopes of cumulative neurobehavioral deficits and physiological changes across days of chronic sleep restriction, to determine the extent to which the duration of sleep per 24 hours (in the range commonly experienced by astronauts in flight) and the use of combined anchor + nap sleep opportunities each day, can prevent or attenuate the development of cumulative fatigue and performance deficits. The response surface experimental paradigm affords a high return of information regarding the optimal way to utilize sleep in operations that inherently limit time for sleep in the space flight environment. The results of the proposed research will contribute to the optimization of performance, productivity, safety and health during extended missions, by providing astronauts with the most efficient sleep-wake schedules. The results of this project also have important implications for optimizing work-rest scheduling in Earth-based safety-sensitive industries that must operate around the clock (e.g., transportation, military, public safety).

Finally, the research project will also help address critical questions pertaining to human performance failure in space due to sleep and circadian problems. Thus, the results of this project will help determine both the acute and long-term neurobehavioral and physiological effects of exposure to restricted sleep durations in the range commonly experienced by astronauts in space flight. It will establish whether sleep-wake schedule countermeasures involving varying combinations of restricted anchor sleep and nap sleep can effectively mitigate the performance risks posed to astronauts by chronically restricted sleep in space flight. The project will also provide estimates of the long-term effects of optimal sleep schedule countermeasures on hormonal profiles, sleep inertia and related physiological and neurobehavioral functions. Finally, the project is providing performance technologies and needed data for the development of a biomathematical model of human performance capability relative to sleep schedules and circadian physiology. These techniques will ultimately aid astronauts in the self-management of sleep and alertness during long-duration space flight.

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I. PROJECT RESEARCH ACTIVITY

A. OVERVIEW AND PRIMARY GOAL OF PROJECT

The performance capability of astronauts during extended-duration space flight depends heavily on achieving daily recovery through adequate sleep. There is considerable evidence that astronaut sleep is restricted in space flight to averages of 4hr—6.5hr/day (M = 6.0hr/day), due to conditions that are expected to be present during long duration missions (e.g., microgravity, circadian displacement of sleep, operational demands, noise, stress). However, ground-based experiments have demonstrated that daily nocturnal sleep durations in this range result in progressive sleepiness, fatigue, cumulative neurobehavioral performance deficits and alterations of certain physiological functions. **The primary goal of this project is to determine how to best prevent such cumulative deficits from sleep restriction by using a response surface experimental paradigm to identify the most effective ways to obtain sleep in order to maintain the high-level cognitive performance functions required for space flight.** As such, the research addresses questions in the area of sleep and circadian rhythm problems within the broader Human Performance and Behavior area of the critical path roadmap. The response surface experimental paradigm affords a high return of information regarding the optimal way to utilize sleep in operations that inherently limit time for sleep. The results of the project will contribute to optimization of performance, productivity, safety and health during extended missions, by providing astronauts with the most efficient sleep-wake schedules. The results will also have important implications for optimizing work-rest scheduling in Earth-based safety-sensitive industries that operate around the clock (e.g., transportation, military, public safety).

The focus of the research has been on determining how variations in sleep duration and its timing relate to the most efficient return of performance and alertness per unit time invested in sleep, in order to establish whether there is a way to optimize performance in the face of restricted sleep during space flight. Although there is evidence that the less sleep obtained, the greater the waking deficits, experiments have found that for acute periods supplementing a reduced anchor sleep period with a nap has the potential to enhance performance, due to the exponential recovery of neurobehavioral functions relative to sleep duration. **During the past 3 years we have been using a response surface experimental paradigm to systematically determine the chronic effects on performance, mood, sleep, circadian physiology and hormones, of 18 sleep-wake schedules that involve restricted nocturnal anchor sleep alone and in combination with varying durations of restricted daytime naps.** The resulting **response surface maps (RSMs)** derived from this dose-response experiment indicate that total sleep time per 24hr is a prime determinant of cumulative neurobehavioral deficits, and that combining a restricted nocturnal anchor sleep with a midday nap can attenuate cumulative deterioration in performance in the face of chronic sleep restriction. After reviewing the background pertinent to the experiment we performed, we describe the specific aims and hypotheses, and the progress we made in addressing these during the past 3 years.

B. BACKGROUND JUSTIFICATION FOR PROJECT

1. Astronaut sleep in space is disrupted and reduced in duration

There is considerable evidence that despite recommendations to astronauts to sleep 8 hours per 24-hr while in orbit,[50,60] astronaut sleep is nearly always restricted in space flight to averages between 4hr/day and 6.5hr/day. **The average sleep obtained in space flight is typically reported to be approximately 6.0 hours per 24-hr period.** This figure has not changed substantially from the earlier studies that relied on large numbers of astronaut self-reports or logs,[29,32,60,66] to the more recent objective polysomnographic results from sleep in space.[33,34,46,67] For example, in a flight debriefing survey, 58 crew members from a total of nine space shuttle missions (ranging in duration from 4 to 9 days) reported

on average 6.03 hours sleep per day while in space, compared to 7.9 hours sleep while on the ground.[60] A recent study by Gundel and colleagues[34] of polysomnographically (PSG) recorded sleep in four astronauts aboard the Russian Mir space station for multiple nights between the 3rd and 103rd day aloft, reported that daily physiological sleep averaged 6.11 hours, which suggests that sleep duration does not increase or normalize to baseline levels as flights become longer. Monk and colleagues[46] confirmed the Mir sleep findings by Gundel and colleagues[33,34] in a U.S. space mission (STS-78). In a study of PSG recorded sleep in four orbiting astronauts aboard STS-78 they recorded an average of 6.06 hours sleep on the 3rd and 12th days of flight, even though the mission was specifically scheduled to minimize disruptions in circadian rhythms and sleep.[46] In summary, virtually every study of sleep in space, and especially recent investigations that recorded physiological sleep (which have included U.S. and European astronauts; sleep on both the Mir space station and the U.S. space shuttle; and flight periods up to 100 days) have reported that during space flight daily sleep durations average approximately 6 hours. In addition, both the studies of Gundel[34] and Monk[46] reported decreased PSG slow wave sleep (SWS) and/or decreased EEG slow wave activity (SWA) on orbit, which contrasts with ground-based studies of sleep restriction in which increases in SWS and SWA were noted.[8,9] This discrepancy suggests that not only is sleep shorter during space flight, but that there may be a disruption of the putative restorative homeostatic component of sleep.[7] Further evidence of sleep disturbances in space flight comes from a recent survey of pharmaceutical use by U.S. astronauts in which it was reported that 45% of all medications taken by 219 astronauts on 79 shuttle missions were hypnotics for sleep disturbances, and that when used "sleep medications were used throughout the mission duration (p. 706)."[53] The report concluded "sleep disturbances are becoming the predominant problem for U.S. Shuttle astronauts. (p. 708)."[53] A recent report also summarized the significant sleep problems experienced by Russian cosmonauts during long-duration missions aboard Mir.[59]

2. Causes of astronaut sleep reductions in space

The causes of disturbed sleep and reduced sleep duration in space flight have been attributed to multiple factors including shift work operations, noise levels, motion sickness, and excitement;[60] to changes in physical activity associated with weightlessness, and circadian rhythm disturbance;[34] to stress;[59] and to ambient temperature, need to void, vibration, mission circumstances, general discomfort, and spontaneous awakening.[46] The fact that Gundel[34] and Monk[46] observed similar SWS disturbances and identical reduced sleep durations in different astronauts in markedly different space craft; on flights of very different durations; and both with and without circadian rhythm alterations,[34,46] suggests that sleep reductions are associated with more persistent features of space flight, such as microgravity effects and operational requirements, rather than with more transient causes (e.g., motion sickness, excitement, noise). **The results to date suggest that reduced sleep will continue to be present during long-duration space missions, and require countermeasures to prevent it from posing risks to astronaut neurobehavioral functioning.** Since it is not clear that sleep duration can be extended in space flight to optimal levels (i.e., 7-8hr/day), it is important to establish whether cumulative deficits from chronically restricted sleep can be prevented or attenuated by supplementing reduced anchor sleep periods with naps. **This project specifically addresses this countermeasure question.**

3. Reduced sleep can adversely affect astronauts in space flight

There is reason to expect that fatigue from inadequate sleep is likely to occur during a long-duration mission, especially if operational demands become chronically elevated. Recent anecdotal evidence from both U.S. and Russian space missions has revealed that astronauts/cosmonauts on longer-duration missions have experienced problems induced by operational demands involving high workload and performance of mission critical tasks.[17,28,42,52,59,61] Problems arose when unexpected and/or underestimated operational requirements occurred while crews were already experiencing work-related stressors, including fatigue from inadequate rest and sleep.[28] In some of these instances, stressed flight crews withdrew from voice communications with ground controllers,[10,61] or when pressed to continue performing, made errors that seriously jeopardized the mission (e.g., 1997 collision of the Mir space

station and Progress 234[17,28]). A recent report from the Russian Space Program discusses performance and psychological problems caused by disturbed and reduced amounts of sleep during long-duration space flights. Consequently, it appears that astronauts are not immune to chronic sleep disruption or to the cumulative adverse effects on neurobehavioral functions of chronic partial sleep loss that might ensue from reduced sleep during long-duration missions. Therefore, it is important to continue to investigate ways to reduce the risks posed by chronic sleep restriction in space flight. **The experiment we performed on sleep timing as a countermeasure to cumulative deficits in space flight is the first to determine the most efficient return of performance and alertness per unit time invested in sleep, for the goal of establishing whether there is a way to optimize performance in the face of restricted sleep during space flight.**

4. Cumulative neurobehavioral deficits develop with chronic sleep restriction

Sleep curtailment is a potentially serious risk for extended duration space flight because sleep is among the most fundamental determinates of cognitive performance and physical resilience.[22] A growing number of ground-based experiments have demonstrated that daily sleep durations in the range commonly reported for astronauts (i.e., 4hr/day to 6.5hr/day) result in progressive sleepiness, fatigue, cumulative neurobehavioral performance deficits and alterations of certain physiological functions.[13,15,26,37,39,40,57,58,62] The mechanism through which this occurs is the development of cumulative homeostatic pressure for sleep across consecutive days of sleep restriction.[13,15,26] The neurobehavioral deficits include increased propensity for sleep, lapses of attention, slowed response times, degradation of complex problem solving, reduced learning, and in some individuals, uncontrolled sleep attacks.[22,23] These cumulative performance deficits are often not accompanied by subjective awareness of the severity of the impairment,[23,40] which may explain why astronauts do not report performance problems when sleep is restricted. Alterations of sleep- and circadian-mediated hormonal profiles have also been observed in subjects exposed to chronic sleep restriction,[48,62] as have metabolic changes.[62] **Our completed experiment is the first ground-based study to utilize the slopes of cumulative neurobehavioral deficits and physiological changes across days of chronic sleep restriction, to determine the extent to which (1) duration of sleep per 24hr (range 4.2hr – 8.2hr); (2) the use of anchor and nap sleeps; and (3) the placement of sleep within the circadian cycle, affect these cumulative changes.**

5. Anchor sleep restriction and napping to reduce cumulative deficits

The use of a single nap each day has the potential to prevent the cumulative effects of chronic sleep restriction during extended duration space flight. This hypothesis is based on a substantial scientific literature on the timing, nature, and effects of nap sleep.[5,19,31,56,65] Polycyclic sleep is common in animals, and both sleep propensity and the likelihood that a nap will occur at midday reflect an inherent biphasic sleep tendency in the human sleep/wake cycle.[11,12,14,19,41,54] Ground-based laboratory and field studies have consistently demonstrated that the likelihood that a person will take a nap increases markedly when the major sleep period (i.e., anchor sleep[44,45] is reduced in duration by 1hr or more, or is ≤ 6.5hr in duration, regardless of whether the person is entrained to a normal circadian day,[19,24] or is working shifts that disrupt sleep.[1,16,55] Consequently, most napping involves "compensatory" or "replacement" sleep in response to an elevated homeostatic drive for sleep.[20] Such naps typically have durations ≤ 2.0 hr, and have consistently been found to result in improved performance and physiological alertness.[19,68] The enhancement of neurobehavioral functions has been especially dramatic when a single nap of 0.4hr to 2.0hr duration was used to promote performance capability during sustained operations involving sleep loss or night work.[4,5,6,25,27,30,31,35,36,43,47,49,51,56,63,64] Subjects assigned to the nap conditions in these studies were able to fall asleep quickly, and following 15-min post-nap recovery from sleep inertia, they performed at levels superior to subjects in control conditions for between 4-12 hr. The use of naps to maintain performance capability in continuous operations involving restriction of anchor sleep periods has been called "prophylactic napping"[25] and "planned napping"[56]

The countermeasure goal we sought to achieve in the RSM experimental test of anchor + nap sleep was the promotion of alertness and performance via time-efficient preemptive relief of accumulating sleep pressure. Since recovery as a function of sleep duration appears to occur exponentially,[3,7,18,38] a nap involves the steepest portion of the recovery curve, making its effects on subsequent performance disproportionately beneficial relative to the time invested in it. Consequently, although scientific evidence strongly supports the view that the less sleep obtained, the greater the likelihood of waking deficits, both laboratory and field experiments have found that for acute periods at least, supplementing a reduced anchor sleep period with a nap has the potential to sustain optimal performance.[2,4,11,21,25,56,65] Thus, the disproportionate recovery potential of naps may be due to the exponential recovery of neurobehavioral performance functions in relation to sleep duration. This exponential process appears to parallel the time course of EEG slow wave activity (SWA obtained by power spectral analysis) during sleep, which is believed to manifest the physiological homeostatic drive for sleep.[7,9] This suggests that the daily implementation of a brief nap may be one way in which cumulative sleep loss and waking performance deficits could be reversed or prevented. However, there is a critical deficiency in knowledge in this area, since nap strategies have not been systematically tested as countermeasures to performance impairment from cumulative anchor sleep restriction. This critical gap in knowledge on how a single daily nap can be most effectively used to eliminate the cumulative effects of chronic sleep restriction prompted the experiment we performed. **This project specifically addressed the issue by developing dose-response RSMs from a wide range of variations in the durations of both anchor sleep and nap sleep, to establish the sleep-wake schedules that most effectively limit the cumulative adverse consequences on performance and physiology of chronic sleep restriction at the levels typically experienced in space flight.**

C. PROGRESS TOWARD SPECIFIC AIMS AND HYPOTHESES

The prevention of cumulative performance deficits from sleep restriction during extended duration space flight involves finding the most effective ways to obtain sleep in order to maintain the high-level cognitive and physical performance functions required for manned space flight. There is a critical deficiency in knowledge of the effects of how variations in sleep duration and timing relate to the most efficient return of performance per unit time invested in sleep during long-duration missions, and how the nature of sleep physiology (i.e., sleep stages, sleep electroencephalographic [EEG] power spectral analyses) change as a function of sleep restriction and performance degradation. The primary aim of this project has been to meet these critical deficiencies through utilization of response surface modeling, testing in a dose-response manner, combinations of sleep duration and timing, for the purpose of establishing how to most effectively limit the cumulative adverse effects on human performance and physiology of chronic sleep restriction. RSM is an efficient way to answer the basic question of whether the desired gains in performance capability occur when total daily sleep time is increased (1) through increasing durations of anchor sleep; (2) through increasing durations of nap sleep; or (3) through increasing durations of anchor + nap sleep. The project has made the following progress on the five specific aims.

1. Progress toward specific aim 1: Establish ways to use sleep to promote neurobehavioral performance in the face of sleep restriction

As discussed in the background, in order to counter cumulative waking deficits from sustained sleep restriction, either the sleep drive must be met through increased duration of the major (anchor) sleep episodes, and/or through the strategic use of a single daily pre-planned (preemptive) nap, which also segments one long waking episode into two shorter durations. However, there is a critical deficiency in knowledge of the effects of varying combinations of anchor sleep and nap durations that will yield the most efficient return of performance per unit time invested in sleep. We designed an experiment that permitted an evaluation of a wide range of anchor sleep + nap sleep combinations and sleep dosages, in order to address specific aim 1 (i.e., to establish ways to use sleep to promote neurobehavioral

performance during chronic sleep restriction). **Using a laboratory protocol in which N = 90 healthy adults were continuously and intensively monitored for 14 consecutive 24-hr periods, combined with a two-stage regression approach, we established a response surface model of the countermeasure effectiveness of 18 different combinations and durations of anchor sleep and scheduled naps to test the hypothesis that the addition of a preemptive midday nap to restricted nocturnal (anchor) sleep each day would attenuate the *slope* (growth) of the cumulative performance deficits developed across days.**

a. Data acquisition is complete. The goal of the project was to complete study of N = 90 healthy adult subjects (females and males) evaluated on different sleep-wake schedules during a 14-day laboratory protocol that simulated aspects of space flight (i.e., small-group confinement, environmental and social isolation, low light levels, controlled activity demands, fixed diet; quasi-continuous performance demands). Intensive physiological monitoring during both waking and sleeping periods was completed throughout the 14 days (i.e., EEG, EOG, EMG, core body temperature, plasma melatonin, cortisol and growth hormone); and quasi-continuous neurobehavioral testing was completed throughout the 14-day period (i.e., activity levels, psychomotor vigilance, cognitive throughout, working memory, multi-tasking performance, learning, frontal lobe function, mood, alertness, subjective symptoms/complaints).

Data acquisition in this project has been completed. A total of 1,783 volunteers were initially screened by telephone interview. A total of 198 (11%) of these respondents were fully qualified at initial interview and then underwent extensive laboratory screening (i.e., blood chemistry, drug screen, psychological and physical exams, and stable sleep-wake cycles by objective actigraphic documentation for 1-2 weeks prior to study). A total of 106 of these subjects were fully qualified for the study by all objective criteria, and they were empanelled into the laboratory protocol, resulting in a grand total of 1,344 laboratory days (24-hr periods) completed. A total N = 91 of the 106 subjects completed the entire 14-day protocol. These 91 subjects satisfied the goal of 90 subjects needed for the response surface modeling. Target goals for age, gender, and racial mix were also met (M = 29.8 years [range 21-49 years]; n = 50 males, n = 41 females; 47% white, 40% black, 11% asian or Hispanic). The 18 conditions and samples sizes used in each condition for generating the overall RSMs are shown in Table 1.

Table 1. The 18 anchor + nap sleep conditions (and sample size evaluated for each) studied experimentally for development of response surface maps on neurobehavioral and physiological variables.

Total time in bed (TIB) per 24hr for each of 18 conditions used in RSM development.				
Duration of **daytime nap** sleep opportunity (hr TIB)	Duration of **nocturnal anchor** sleep opportunity (hr TIB)			
	4.2	5.2	6.2	8.2
0	4.2 (n = 5)	5.2 (n = 5)	6.2 (n = 5)	8.2 (n = 6)
0.4	4.6 (n = 5)	5.6 (n = 5)	6.6 (n = 5)	*
0.8	5.0 (n = 5)	6.0 (n = 5)	7.0 (n = 5)	*
1.2	5.4 (n = 5)	6.4 (n = 5)	7.4 (n = 5)	*
1.6	5.8 (n = 5)	6.8 (n = 5)	*	*
2.0	6.2 (n = 5)	7.2 (n = 5)	*	*
2.4	6.6 (n = 5)	*	*	*

*condition not tested

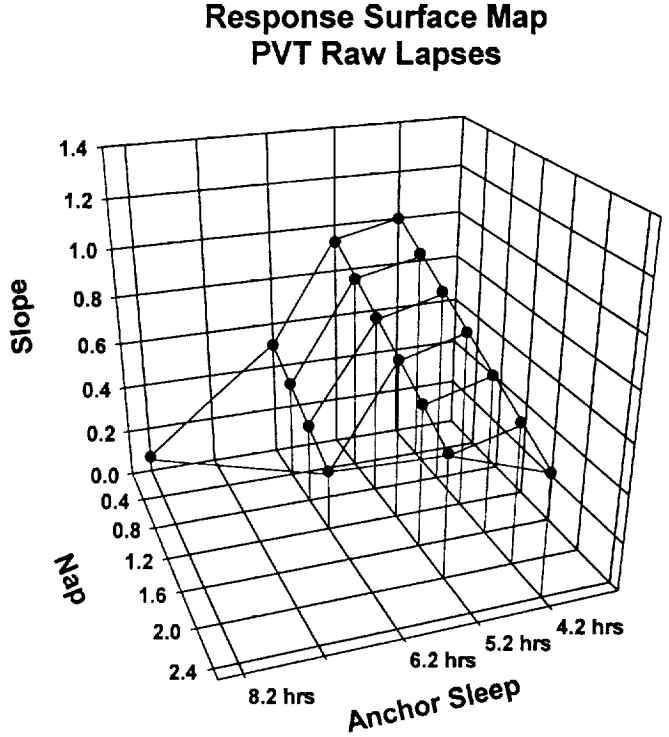
As displayed in Table 1, n = 5 subjects were studied for each of the 18 conditions, except for the control condition of 8.2 hr anchor time in bed (TIB) with no nap (n = 6). It is important to note that these cell sample sizes were the target goal based on power calculations. The total planned sample size for the experiment was n = 90. The power characteristics of the overall null hypothesis were assessed. We first assumed that 5% of the variance in the outcome measure could be accounted for by a linear regression model containing only gender + age + age-squared. At least 80% power to reject the overall null

hypothesis of no variance accounted for by adding the anchor sleep and nap factors is achieved as long as the increase in the explained variance (R-square) is at least equal to 17%, resulting in a total model R-square of 22% (using an F-test with df = 11,75). If the gender/age model explains no variance, then the R-square for the anchor sleep/nap conditions must be at least 18%. Thus, the power of our study depends on approximately 1/5 of the variance in the rate of the increase in cumulative functional deficits to be explainable by the experimental factors under study. Response surface modeling of neurobehavioral variables on the data set shows that this minimum explainable variance due to sleep conditions was achieved.

b. Response surface modeling of neurobehavioral data. As of September 29, 2000, response surface model development and hypothesis testing on the large set of neurobehavioral and physiological outcomes was underway. Random coefficients regression models are being used to estimate subject-specific mean decrements associated with cumulative exposure to the 18 chronic sleep ration protocols for primary performance, subjective, and physiological variables. Neurobehavioral values subjected to linear regression were means over time-of-day values (i.e., 07:00 to 23:30 hours) excluding sleep inertia bouts—which are being analyzed separately to determine the "impairment cost" of sleep schedules. Slopes were estimated using two-stage linear regression from baseline to experimental schedule day 8. Days 9 and 10 were excluded because subjects did not have evaluations at all circadian times in order to provide growth hormone blood samples. These subject-specific slopes were then used in response surface modeling in order to identify optimal nocturnal anchor sleep + daytime nap combinations that minimize adverse effects on neurobehavioral and physiological functions from chronic sleep restriction. The spatial locations of optimal solutions were also being graphically illustrated by plotting the expected slope as a function of the response surface model. All response surface models being evaluated include the following factors when attempting to optimize the benefits of reduced sleep opportunities: (1) nocturnal anchor sleep duration, (2) midday nap sleep duration, (3) age, (4) gender, and (5) baseline individual differences. An anchor sleep by nap interaction was first assessed, and if not significant, a model that included squared terms for nap duration and age was evaluated. These latter two were then removed one-at-a-time if not significant. Thus, when there is no interaction the final RSM was selected using either the actual response value (i.e., two-stage regression with intercept) or the change from baseline value (i.e., two-stage regression without intercept). In general, we have found that objective performance variables were best evaluated using the actual response/with intercept first stage model, while subjective response variables were best evaluated using the change from baseline/no intercept first stage models. **Thus far analyses are nearly complete on a wide range of performance and subjective, to test the hypothesis that the addition of a preemptive midday nap to restricted nocturnal (anchor) sleep each day would attenuate the *slope* (growth) of the cumulative performance deficits developed across days (see Figures 1 and 2, and Table 2 in Appendix A).** The resulting RSMs reveal that the addition of a daytime nap to limited anchor sleep will reduce the cumulative performance deficits and subjective fatigue that develops with chronic sleep restriction. The response surface modeling has also revealed potentially important effects of age and gender on these cumulative functions.

As illustrative examples of the results of the modeling relative to the hypothesis in specific aim 1, Figures 1 and 2 display the RSMs that optimally characterized a primary performance variable—psychomotor vigilance test (PVT) lapses—and a primary subjective sleepiness variable—Karolinska Sleepiness Scale (KSS). Figures 1 and 2 clearly illustrate that elevated slope in performance lapses and sleepiness (i.e., deficit functions) that develop when anchor and nap sleep durations are restricted. Development of optimum response surface models for other key neurobehavioral performance and subjective outcomes are summarized in Table 2 (see Appendix A).

Figure 1. Response surface map (RSM) fit to PVT performance lapses and derived to reflect the change in lapse frequency across days of sleep restriction (i.e., slope) as a function of anchor and nap sleep durations, of a typical healthy 30-yr old male with baseline performance equal to the grand average for the group. The RSM model shown was the optimal second-stage fit to the data resulting from the set of first stage regressions (i.e., slope of lapses across days of sleep restriction for the 18 experimental conditions studied). The RSM is the product of regression model 2B (absolute value = first stage regression with intercept), which accounted for an adjusted R^2 = 28% of the variance ($F = 7,78$, $p < 0.0001$). The elements of model 2B are shown below the RSM, and include nocturnal anchor sleep TIB ("Anchor Sleep"), diurnal nap TIB ("NAP"), age, gender, and baseline inter-subject differencs ("Day 0"). The main effect for nocturnal anchor sleep in this model was significant ($F = 3.87$, $p = 0.012$), indicating that the greater the nocturnal anchor TIB, the lower the cumulative increase in PVT lapses across days. Increasing diurnal nap duration also tended to reduce slope increments in performance lapses across days ($F = 3.25$, $p = 0.075$). The significant Day 0 effect indicates that there were differences among subjects at baseline in PVT performance. Age and gender made no substantive contribution to variability in PVT performance lapses across days.

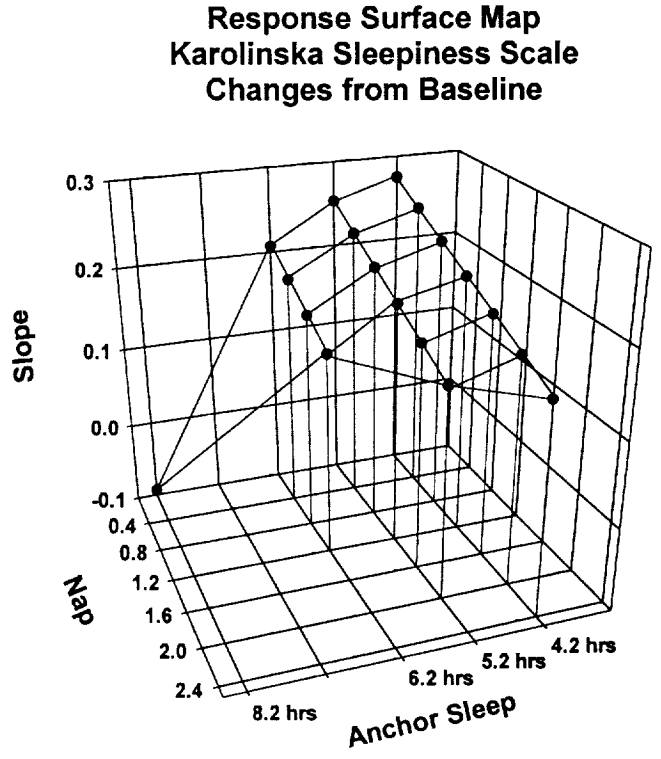


RSM Model: Adjusted R^2=28.0%, F=7,78, p<0.0001

E(Slope) = f([Intercept], [Anchor sleep indicators], [NAP], [(Day 0-mean(Day 0))], [Age-mean(Age)], [Female Gender])

Anchor Sleep: F=3.87, df=3,78, p=0.012
NAP: F=3.25, df=1,78, p=0.075
Day 0: F=25.1, df=1,78, p<0.0001
Age: F=0.02, df=1,78, p=0.887
Gender: F=1.75, df=1,78, p=0.189

Figure 2. Response surface map (RSM) fit to KSS subjective sleepiness ratings and derived to reflect the change in sleepiness ratings across days of sleep restriction (i.e., slope) as a function of anchor and nap sleep durations, of a typical healthy 30-yr old male with baseline performance equal to the grand average for the group. The RSM model shown was the optimal second-stage fit to the data resulting from the set of first stage regressions (i.e., slope of lapses across days of sleep restriction for the 18 experimental conditions studied. The RSM is the product of regression model 2C (change from baseline = first stage regression without intercept), which accounted for an adjusted R^2 = 33% of the variance (F = 8,77, p < 0.0001). The elements of model 2C are shown below the RSM, and include nocturnal anchor sleep TIB ("Anchor Sleep"), diurnal nap TIB ("NAP"), age, aged-squared (i.e., quadratic age component), gender, and baseline inter-subject differences ("Day 0"). The main effect for nocturnal anchor sleep in this model was significant (F = 7.06, p = 0.0003), indicating that the greater the nocturnal anchor TIB, the lower the cumulative increase in KSS sleepiness ratings across days. Increasing diurnal nap duration also tended to reduce slope increments in sleepiness across days (F = 3.66, p = 0.059). The significant Day 0 effect indicates that there were differences among subjects at baseline in KSS sleepiness ratings. Unlike the effects of sleep restriction on PVT performance lapses, however, age (F = 5.42, p = 0.006) and gender (F = 3.04, p = 0.085) also contributed significantly to KSS variability.



RSM Model: Adjusted R^2=33.0%, F=8,77, p<0.0001

E(Slope) = f([Intercept], [Anchor sleep indicators], [NAP], [(Day 0-mean(Day 0))], [Age-mean(Age)], [(Age-mean(Age))^2] [Female Gender])

Anchor Sleep: F=7.06, df=3,77, p=0.0003
NAP: F=3.66, df=1,77, p=0.059
Day 0: F=23.4, df=1,74, p<0.0001
Age: F=5.42, df=2,77, p=0.006
Gender: F=3.04, df=1,77, p=0.085

2. Progress toward specific aim 2: Determine the relationship between sleep physiology and waking performance

Data are currently being analyzed to systematically characterize the nature of cumulative changes in anchor and nap sleep physiology (i.e., sleep latency, total sleep time, sleep efficiency, sleep stages, EEG power spectral analysis for SWA, sleep consolidation), as performance is assessed across days of cumulative sleep restriction for different combinations of anchor sleep and nap sleep duration. These physiological data will yield three important outcomes: (1) they will help establish the extent to which homeostatic physiological processes during sleep respond to chronic sleep restriction; (2) they will permit evaluation of the changes in sleep structure in relation to waking neurobehavioral deficits; and (3) they will provide information on how different sleep schedules (varying anchor + nap sleep durations and timing) affect sleep physiological responses. **We hypothesize that both slow wave sleep (SWS) and rapid eye movement (REM) sleep homeostasis will be critical to maintaining neurobehavioral functions during chronic sleep restriction.** Progress toward testing this hypothesis is being made. The major discovery thus far is that subjects were able to achieve significant levels of physiological sleep in both anchor (nocturnal) and nap (diurnal) sleep opportunities across the 10-days of restriction, regardless of the time in bed allowed for sleep. Even daytime nap opportunities as brief as 0.4 hr (24 minutes) consistently resulted in physiological sleep. Data in Tables 3, 4 and 5 of Appendix A illustrate this observation. They also show that as time in bed for sleep was reduced, sleep efficiency increased; and that proportions of SWS and REM sleep were well preserved in many nap conditions. Our plan is to ultimately generate response surface models that include total sleep time and sleep efficiency, as defined from PSG, to account for variation in total amounts of sleep actually obtained in each condition.

3. Progress toward specific aim 3: Explore hormone secretion in relation to anchor and nap sleep durations

In addition to evaluating the polysomnograhic and neurobehavioral responses to chronically restricted anchor + nap sleep, we sought to explore whether the addition of a nap to anchor sleep alters the normal secretory profiles of the sleep- and circadian-mediated hormones melatonin, cortisol, and growth hormone (hGH). Endogenous secretion of melatonin and cortisol is circadian mediated and therefore serves as a marker for the effects of restricted nocturnal anchor sleep and diurnal naps on circadian phase. Endogenous secretion of growth hormone—the majority of which occurs during slow wave sleep—has an important role in the maintenance of bone, cartilage, and muscle; exerts regulatory influence over liver proteins; and is involved in immune and reproductive systems. These critical functions for hGH in prolonged space flight, where prevention of muscle and bone loss is a goal, make it important to ensure that the anchor + nap sleep schedules that optimize performance do not disrupt the normal sleep-mediated release of hGH. In addition, the tight association between hGH release and sleep presents an important opportunity to explore augmenting total hGH output by supplementing the major nocturnal sleep period with daily nap sleep.[71,72] It is particularly important to determine whether naps can enhance hGH secretion in the face of cumulative sleep deprivation, since in adults undergoing acute nocturnal sleep restriction, hGH release is suppressed.[48,73,74,75] To explore the question of hormonal responses to anchor + nap sleep restriction, we collected plasma from an indwelling venous catheter every 15 minutes for 25 hours during the baseline and on the 9th through the 10th day of sleep restriction in the RSM experiment. Blood was assayed for melatonin, cortisol and growth hormone levels using standard procedures. The quantitative analyses of these results are still underway as of 9-29-00. Preliminary data presented in Appendix A (Figures 5, 6 and 7) illustrate the fact that once these analyses are complete we will be able to determine what effects various anchor + nap sleep combinations have had on the endogenous secretory profiles of melatonin, cortisol and growth hormone.

4. **Progress toward specific aim 4: Integrate performance results into a multidimensional mathematical model**

We have been providing neurobehavioral data from our experiments on the cumulative effects of sleep restriction and on naps as a countermeasure to the effects of elevated homeostatic sleep drive, to Drs. Megan Jewett, Richard Kronauer, Emory Brown and Charles Czeisler at Harvard (i.e., NSBRI Human Performance, Sleep and Chronobiology Team projects 1 and 4), for development of a mathematical model on the control of performance by interaction of sleep and circadian systems. This collaboration has proven fruitful.[38,70] Ultimately the development of such a model could help identify the optimal timing of work-rest schedules for astronauts, as well as the timing of countermeasures for prevention of performance-impairing fatigue in long-duration space flight.

5. **Progress toward specific aim 5: Explore the reliability of performance vulnerability to sleep deprivation**

In exploratory analyses, we have tested the hypothesis that a substantial proportion of the differences in response to sleep deprivation among individuals is due to trait-like differential vulnerability. If this hypothesis is supported, it would suggest that it might be possible to retrospectively develop a probed performance algorithm that can be used to prospectively predict individual vulnerability to cumulative sleep loss. The effects of sleep deprivation on sensitive tasks like psychomotor vigilance test (PVT) performance are typically associated with a proportionality between the mean and standard deviation,[69] which results from a marked increases in inter-subject variability in response to sleep loss (i.e., up to an order of magnitude in lapses of attention during performance). In other words, while a subset of subjects show dramatic performance failures after as little of 16 hr awake, others show almost no performance changes until approximately 42 hr awake. In a separate experiment on the replicability of individual differences in the magnitude of responses to acute sleep deprivation, we have preliminary evidence to support a **hypothesis** that a substantial portion of the variance—perhaps as high as 59%—in the performance response to sleep loss is trait-like differential vulnerability. To more thoroughly address this important question, we have obtained separate funding (Dr. Van Dongen, P.I.) for a project that will systematically expoised subjects to sleep loss across replications that vary state conditions, in an effort to establish (1) whether extremes in response to sleep loss occur even when subjects have the same sleep/wake histories; (2) determine the magnitude of the response to sleep loss is reliably reproducible; and (3) whether predictors of this hypothesized vulnerability can be identified from a range of psychological, behavioral, and biological candidates. If it is possible to establish that performance vulnerability to sleep loss is trait-like and detectable in subjects before sleep deprivation develops, then a "vulnerability algorithm" will be constructed and prospectively tested in future research. If we can predict neurobehavioral vulnerability to cumulative sleep loss, the algorithm will offer a way to more precisely target countermeasures for prevention of performance impairment from chronic sleep loss during extended duration space flight.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

The performance capability of astronauts during extended-duration space flight depends heavily on achieving daily recovery through adequate sleep. Studies in astronauts using daily logs, actigraphs and polysomnography provide convergent evidence that astronaut sleep is restricted in space flight to averages between 4 hr per day and 6.5 hr per day (mean = 6.0 hr per day). There appear to be multiple causes for the restriction, including endogenous disturbances of sleep (microgravity, motion sickness, stress, circadian rhythms), environmental disruptions of sleep (noise, temperature, light), and curtailment of sleep due to the extended work demands that often accompany space flight operations. An average of 6 hr sleep per day poses a risk to astronauts. Ground-based experiments in multiple

laboratories have demonstrated that daily nocturnal sleep durations in the range of 4 – 6.5 hours result in progressive fatigue, as well as cumulative neurobehavioral performance deficits that include lapses of attention, slowed response times, reduced cognitive throughput, degradation of complex problem solving, impaired learning, and alterations of physiologic functions. Research has shown that these cumulative performance deficits are often not accompanied by full subjective awareness of the severity of the impairment, which may explain why astronauts may not report problems when sleep is restricted. Alterations of sleep- and circadian-mediated hormonal profiles and metabolic changes have also been observed in subjects exposed to chronic sleep restriction.

To counter cumulative waking deficits from sustained sleep restriction, either the sleep drive must be met through increased duration of the major (anchor) sleep episode, and/or through the strategic use of a single daily pre-planned (preemptive) nap, which also segments one long waking episode into two shorter durations. However, there is a critical deficiency in knowledge of the effects of varying combinations of anchor sleep and nap durations that will yield the most efficient return of performance per unit time invested in sleep. The primary aim of this project is to meet this critical deficiency through use of a ***response surface experimental paradigm***, testing in a dose-response manner 18 different combinations of anchor sleep and nap sleep durations for the purpose of establishing how to most effectively limit the cumulative effects of chronic sleep restriction in space operations. This approach is allowing us to test the hypothesis that the addition of a nap to anchor sleep will yield normal neurobehavioral functions and normal secretion of sleep- and circadian-mediated hormones (melatonin, cortisol, growth hormone), even though total sleep per 24-hr is restricted. The experiment is the first ground-based study to utilize the slopes of cumulative neurobehavioral deficits and physiological changes across days of chronic sleep restriction, to determine the extent to which the duration of sleep per 24 hours (in the range commonly experienced by astronauts in flight) and the use of combined anchor + nap sleep opportunities each day, can prevent or attenuate the development of cumulative fatigue and performance deficits. The response surface experimental paradigm affords a high return of information regarding the optimal way to utilize sleep in operations that inherently limit time for sleep in the space flight environment. The results of the proposed research will contribute to the optimization of performance, productivity, safety and health during extended missions, by providing astronauts with the most efficient sleep-wake schedules. The results of this project also have important implications for optimizing work-rest scheduling in Earth-based safety-sensitive industries that must operate around the clock (e.g., transportation, military, public safety).

Thus far, the results of the experiment have revealed that subjects are able to achieve significant levels of physiological sleep when both anchor (nocturnal) and nap (diurnal) sleep opportunities are chronic (i.e., part of a daily schedule), regardless of the time in bed allowed for sleep. Even daytime nap opportunities as brief as 0.4 hr (24 minutes) consistently resulted in physiological sleep. This indicates that the diverse range of restricted anchor + nap sleep durations tested in this protocol will likely result in sleep if used by astronauts. Response surface models fit to neurobehavioral data reveal that the combination of a restricted duration anchor sleep and a diurnal nap can help prevent the development of cumulative deficits that can occur when only restricted anchor sleep is permitted (as is currently typical in space flight). Moreover, all response surface models being evaluated for optimizing performance, mood, sleep physiology, and hormonal profiles in the face of restricted total sleep time include the duration of nocturnal anchor sleep, the duration of diurnal nap sleep, age, gender, and baseline individual differences. Thus, the results of this study will permit us to estimate the relative contributions of astronaut demographics (age, gender) and individual abilities (baseline differences) to cumulative neurobehavioral deficits due to sleep restriction. This permits a more precise estimate of how a given sleep-wake schedule will likely affect different crews.

The research project also has substantial impact toward addressing all four critical path questions pertaining to human performance failure in space due to sleep and circadian problems. Thus,

the results of this project will help determine both the acute and long-term neurobehavioral and physiological effects of exposure to restricted sleep durations in the range commonly experienced by astronauts in space flight (which relevant to critical path question 6.05). It will establish whether sleep-wake schedule countermeasures involving varying combinations of restricted anchor sleep and nap sleep can effectively mitigate the performance risks posed to astronauts by chronically restricted sleep in space flight (which relevant to critical path question 6.06). The project will also provide estimates of the long-term effects of optimal sleep schedule countermeasures on hormonal profiles, sleep inertia and related physiological and neurobehavioral functions (which is relevant to critical path question 6.07). Finally, the project is providing performance technologies (such as the PVT) and needed data to collaborators at Harvard for the development of a biomathematical model of human performance capability relative to sleep schedules and circadian physiology (which is relevant to critical path questions 6.08 and 6.21, respectively). These techniques will ultimately be operationally available to astronauts, to aid them in the self-management of sleep and alertness during long-duration space flight.

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APPENDIX A
Project Research Data

A. ADDITIONAL DATA RELEVANT TO SPECIFIC AIMS

1. Response surface modeling of neurobehavioral variables (specific aim 1).

Findings from the best-fitting RSM for PVT lapses (Figure 1) found no significant contribution from age or gender, but clearly demonstrated a significant difference in neurobehavioral performance between nocturnal anchor sleep durations. As daily anchor sleep duration was decreased from 8.2 hr, to 6.2 hr, to 5.2 hr, to 4.2 hr time in bed each night, there were systematic increases in PVT lapses. In parallel, as the duration of daily time in bed for a nap decreased from 2.4 hr, to 2.0 hr, to 1.6 hr, to 1.2 hr, to 0.8 hr, to 0.4 hr, to 0.0 hr, lapses increased. There was no interaction between anchor and nap sleep durations. PVT lapses are among the most sensitive neurobehavioral metrics of wake state instability due to sleep loss.[20,22,69] In a studies of 7-days of chronic sleep restriction,[26] average daily PVT lapses correlated highly with average daily multiple sleep latency test values ($r = -0.95$, $p < 0.0001$).

Similarly, the response surface map for the KSS sleepiness scores (Figure 2) demonstrated a significant difference in subjective sleepiness levels between the anchor sleep durations, with a greater degree of cumulative subjective sleepiness reported as both anchor and nap sleep durations shortened. Thus, both a representative performance metric (PVT) and a representative subjective metric (KSS) revealed that systematic reductions in daily total time in bed per 24 hours across the 18 sleep-wake schedule conditions resulted in does-response cumulative deficit slopes. This finding confirms and extends evidence that daily sleep durations in the range typically experienced by astronauts (i.e., 4-6 hr/day) result in cumulative neurobehavioral deficits. Ongoing RSM analyses and hypothesis testing are underway on a wide range of additional neurobehavioral, endocrine and physiological variables.

Table 2 (below) summarizes the RSM results other key performance and subjective variables in response to the 18 different sleep restriction conditions. For the sake of comparison among outcomes, the same RSM model (i.e., model 2C) was used for all measures (in most cases it was the optimal model solution). RSM model 2C included the following elements: (1) nocturnal anchor sleep TIB; (2) diurnal nap sleep TIB; (3) age (linear component); (4) age^2 (quadratic component); (5) gender; and (6) baseline inter-subject differences ("Day 0").

Table 2. RSM results for model 2C.

Neurobehavioral Variable	RSM model ANOVA F (8,77)	adjusted R^2	anchor sleep F (3,77)	nap sleep effect F (1,77)	Age effect F (2,77)	gender effect F (1,77)	baseline effect F (1,77)
digit symbol substitution (DSST) performance	p = 0.0008	21.0 %	p = 0.017	p = 0.004	Ns	p = 0.008	ns
psychomotor vigilance performance (1/RT)	p = 0.0011	20.3 %	p= 0.0001	p = 0.031	Ns	ns	ns
post-performance test alertness (subj. scale)	p = 0.0001	38.7 %	p = 0.044	p = 0.028	Ns	ns	p =0.0001
sharp vs. mentally exhausted (subj. scale)	p = 0.0001	42.9 %	p =0.0007	p = 0.015	p = 0.006	p = 0.026	p =0.0001
fresh as a daisy vs. tried to death (subj. scale)	p = 0.0001	38.8 %	p =0.0001	p = 0.009	p = 0.022	ns	p =0.0001
effort to stay awake when not performing (subj. scale)	p = 0.0017	19.0 %	p = 0.006	Ns	p = 0.043	ns	p = 0.003

As is evident from the summarized results of Table 2—and consistent with comparable RSM results for RVT performance lapses and KSS sleepiness ratings shown in Figures 1 and 2, respectively—the effects of varying anchor sleep durations and of varying nap sleep durations were generally statistically significant for key performance and alertness variables. Reductions in nocturnal anchor sleep duration (TIB) resulted in a greater rate of cumulative performance impairment and fatigue. Similarly, reductions in diurnal nap sleep durations (TIB) resulted in a greater rate of cumulative performance impairment and fatigue. Conversely, longer anchor sleep and longer naps yielded smaller cumulative deficits (slopes) across days. These surface maps will also be recalculated based on actual physiological sleep obtained, once all polysomnograhic data are scored, in order to determine whether sleep duration per se were associated with differential rates of deterioration across days. The goal is to identify those restricted sleep (anchor + nap) conditions that yielded the shallowest slopes in cumulative deficits.

Age effects and baseline individual differences were also evident for many of the subjective ratings of tiredness. The age effect reflected the fact that fatigue increased more rapidly across days of sleep restriction as age increased. Significant baseline individual differences were frequently evident in subjective estimates of tiredness, sleepiness, and fatigue. These likely reflect differential use of the psychometric scales by subjects. The effect of gender was evident in DSST performance and ratings of mental sharpness, with female gender being associated with less exhaustion and better cognitive throughput performance (items correct per minute) on the DSST, controlling for experimental condition (i.e., duration of anchor and nap sleep), for age, and for baseline differences. In other words, the average daily per trial change in correct responses (i.e., learning on the DSST) was more positive by 0.43 responses among females compared to males, which attenuated the decline in female performance in the most sleep deprived conditions. **These data serve to illustrate the power of the RSM modeling to estimate not only the relative contributions of anchor and nap sleep durations to cumulative neurobehavioral deficits, but also to yield quantitative estimates of the relative contributions of age, gender, and individual differences to these effects.**

2. Illustrative results of stage 1 regressions (specific aim 1).

Figures 3 and 4 further explore the benefits of adding diurnal naps to nocturnal anchor sleeps, by showing the stage 1 linear regression analyses for PVT lapses (Figure 3) and KSS sleepiness ratings (Figure 4). For these analyses, regression lines were fit to average lapses and KSS ratings. The results show an interesting dose-response relationship between time in bed and the rate of lapse increment (slope) across days of sleep restriction. In the control condition of 8.2 hr TIB + no nap (Figure 3A), PVT lapse averages were low across days and show no signs of cumulative increases (i.e., zero slope = 8.2hr TIB prevented cumulative impairment). In contrast, the 4.2hr anchor + 0.4hr nap TIB (Figure 3F) displayed a clear positive slope across days of sleep restriction, which is evidence for escalating dysfunction. Similar, albeit less severe slopes were seen for average performance deterioration rates for the 5.2hr anchor + 0.4hr nap TIB (Figure 3E) and for the 6.2hr + 0.4hr nap TIB (Figure 3C) conditions. However, when nap duration TIB was increased to 1.2hr and 2.4hr, lapse slopes became shallow (Figures 3B and 3D). Especially interesting are the results for the 4.2hr anchor + 2.4hr nap TIB (Figure 3D) relative to the 6.2hr anchor + 0.4hr nap TIB (Figure 3C). Both conditions limited total sleep per day to 6.6hr TIB (see Table 3, which shows they also yielded similar average daily total sleep times [347.9min., 358.9min.], similar sleep efficiencies [87.9%, 90.6%], and similar sleep latencies [13.3 min., 17.0 min.]), but their effects on PVT lapses were markedly different. The longer daytime nap of the nocturnal anchor 4.2hr + 2.4hr nap TIB condition appeared to prevent the escalation (positive slope) of lapses across days (Figure 3D), while consolidation of the same amount of total daily TIB confined primarily the nocturnal (anchor) period did not prevent the accumulation of performance impairment (Figure 3C). In contrast to the

PVT performance results, stage 1 linear regression analyses of average KSS sleepiness ratings revealed few differences in slopes (Figure 4A-F). Instead, subjective ratings of sleepiness appeared to differ among conditions based on mean level (y-intercept). For example, in the control condition (Figure 4A) of 8.2hr anchor + no nap, KSS ratings remained relatively low overall compared to those for the 4.2hr anchor + 0.4hr nap TIB condition (Figure 2F). We have also seen shallower slopes for subjective data than for performance results in previous experiments on chronic sleep restriction that did not involve naps.[26] These results help clarify the results of the RSMs for PVT lapses and KSS ratings, and they suggest that some combination of shorter nocturnal anchor sleep and longer daytime nap sleep may be preferable to an equivalent sleep time involving anchor sleep only.

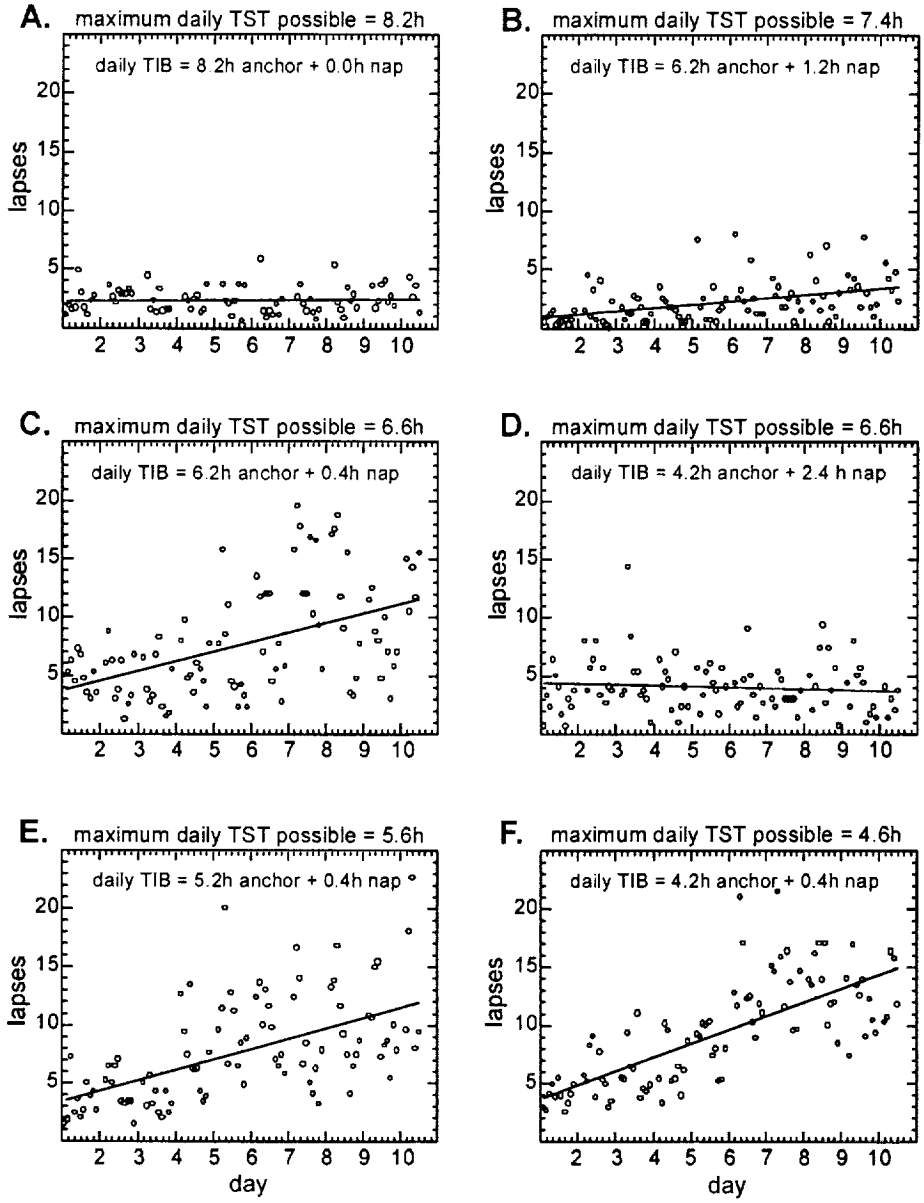


Figure 3 (graphs A, B, C, D, E, F). Psychomotor vigilance test (PVT) performance lapses as a function of six sleep-wake experimental conditions, each maintained for 10 consecutive days, in a controlled, low-light laboratory environment. Each graph is labeled with the maximum total sleep time (TST) possible per day, and the duration of the time in bed (TIB) anchor sleep opportunity (nocturnal) and nap sleep opportunity (diurnal) each day. Graphs display average data (4-7 subjects per graph) for all PVT test times (tests occurred every 2 hours subjects were awake). Linear regression slopes fit to average data are shown as solid lines across the 10 days of testing.

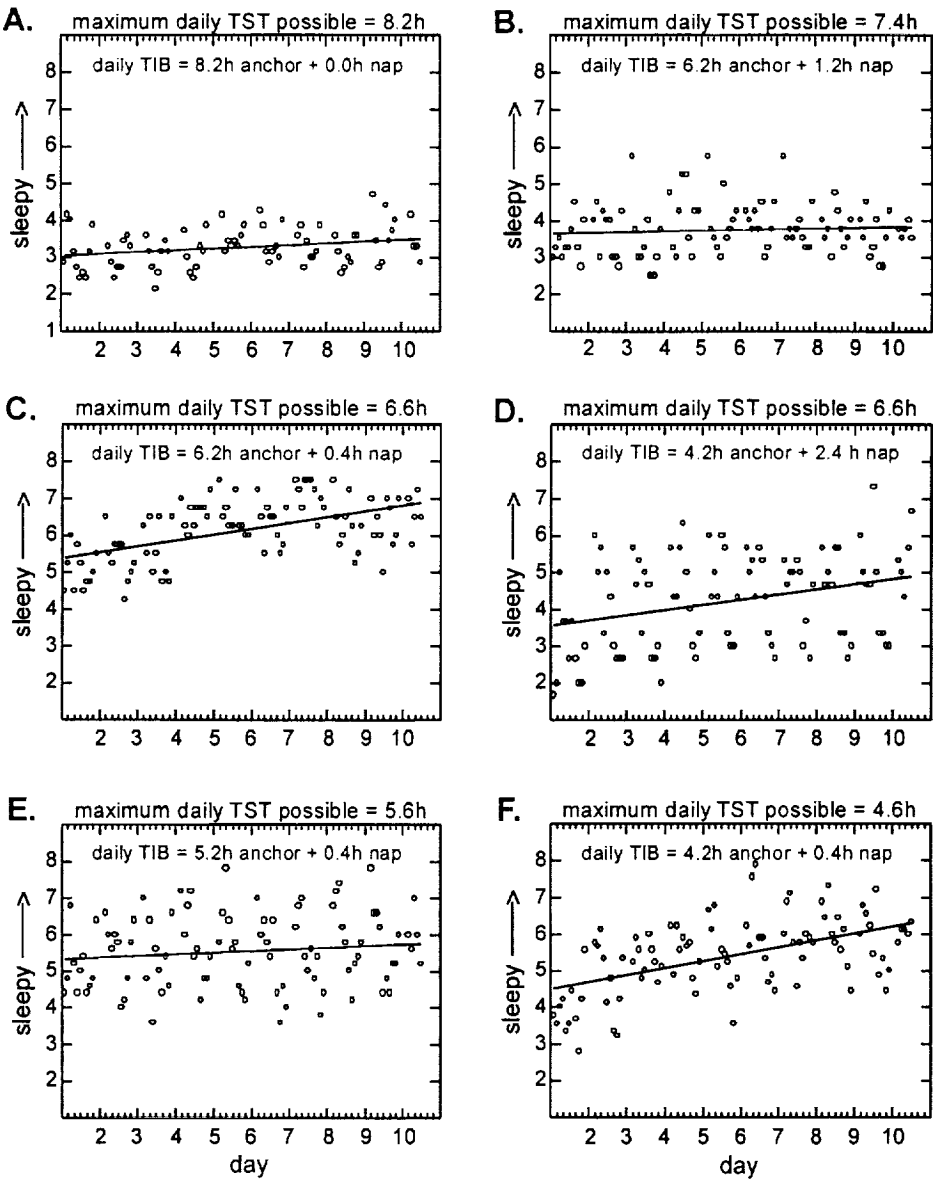


Figure 4 (graphs A, B, C, D, E, F). Karolinska Sleep Scale (KSS) ratings as a function of six sleep-wake experimental conditions, each maintained for 10 consecutive days, in a controlled, low-light laboratory environment. Each graph is labeled with the maximum total sleep time (TST) possible per day, and the duration of the time in bed (TIB) anchor sleep opportunity (nocturnal) and nap sleep opportunity (diurnal) each day. Graphs display average data (4-7 subjects per graph) for all KSS ratings during tests bout every 2 hours subjects were awake. Linear regression slopes fit to average data are shown as solid lines across the 10 days of testing.

3. Anchor and nap sleep physiology (specific aim 2).

Polysomnographic (PSG) data are currently being scored and analyzed. This is a large data set that is comprised of 1,183 nocturnal anchor sleeps (including baseline, sleep restriction and recovery nights), and 700 diurnal naps. As of 9-29-00, data for 10 of 18 sleep restriction conditions have been scored (i.e., 56% completed). Tables 3, 4 and 5 display selected aspects of PSG results. Table 3 shows average total sleep per 24hr (i.e., nocturnal anchor sleep + diurnal nap sleep) across days of sleep restriction. Table 4 shows averages for nocturnal anchor sleeps, and Table 5 shows averages for diurnal naps. These results confirm that subjects were able to sleep in all conditions and even during the briefest nap opportunities (i.e. 0.4hr nap TIB). Although the 8.2hr anchor sleep (control) condition had the least efficient sleep (Tables 3, 4), it was quite effective in preventing cumulative deficits (Figures 1, 2, 3, 4).

Table 1. Average daily total sleep time per 24 hr. (i.e. nocturnal sleep + diurnal nap sleep). Conditions ordered by total time in bed per 24hr, from shortest to longest.

Nocturnal anchor TIB (hr)	4.2	4.2	4.2	5.2	4.2	5.2	4.2	5.2	6.2
Diurnal nap TIB (hr)	0	0.4	0.8	0	1.2	0.4	1.6	0.8	0
Total TIB (h) per 24 hr	**4.2**	**4.6**	**5.0**	**5.2**	**5.4**	**5.6**	**5.8**	**6.0**	**6.2**
Total Sleep Time (min)	^{L}237.8	243.4	**	271.0	289.6	308.0	**	**	329.5
Sleep Efficiency (%)	^{H}94.4	88.2	**	86.9	89.4	91.7	**	**	88.6
Sleep Latency (min)	11.4	^{L}6.8	**	19.4	16.0	13.6	**	**	25.1

Nocturnal anchor TIB (hr)	4.2	5.2	4.2	6.2	5.2	6.2	5.2	6.2	8.2
Diurnal nap TIB (hr)	2.0	1.2	2.4	0.4	1.6	0.8	2.0	1.2	0
Total TIB (h) per 24 hr	**6.2**	**6.4**	**6.6**	**6.6**	**6.8**	**7.0**	**7.2**	**7.4**	**8.2**
Total Sleep Time (min)	312.8	**	347.9	358.9	**	**	**	**	^{H}404.8
Sleep Efficiency (%)	84.1	**	87.9	90.6	**	**	**	**	^{L}82.3
Sleep Latency (min)	26.3	**	17.0	13.3	**	**	**	**	^{H}34.2

* The condition with the highest mean value and the condition with the lowest mean value for each variable are indicated: ^{H}highest value(s) among the 10 conditions, scored as of 09-29-00; ^{L}lowest value(s) among the 10 conditions, scored as of 09-29-00.

** Conditions where polysomnographic scoring of the data has not been completed as of 09-29-00.

Table 2. Average nocturnal anchor sleep time per 24 hr. on sleep restriction days. Conditions ordered by total time in bed per 24hr, from shortest to longest.

Nocturnal anchor TIB (hr)	**4.2**	**4.2**	**4.2**	**5.2**	**4.2**	**5.2**	**4.2**	**5.2**	**6.2**
Diurnal nap TIB (hr)	0	0.4	0.8	0	1.2	0.4	1.6	0.8	0
Total TIB (h) per 24 hr	4.2	4.6	5.0	5.2	5.4	5.6	5.8	6.0	6.2
Total Sleep Time (min)	237.8	224.0	**	271.0	224.8	292.8	**	**	329.5
Sleep Efficiency (%)	H94.4	88.9	**	86.9	89.2	93.8	**	**	88.6
Sleep Latency (min)	11.4	L9.4	**	19.4	24.7	17.5	**	**	25.1
% WASO + movement time	4.4	5.4	**	6.9	4.9	L3.6	**	**	8.7
% NREM sleep	H73.7	73.1	**	63.8	65.7	64.8	**	**	L57.6
% Stage 1 sleep	L2.4	3.7	**	2.8	3.9	3.9	**	**	4.9
% Stage 2 sleep	47.8	49.0	**	42.0	35.3	L34.6	**	**	48.4
% Slow wave sleep (SWS)	23.5	20.4	**	19.0	H26.5	26.3	**	**	14.3
% REM sleep	18.0	20.0	**	H22.3	20.5	22.1	**	**	18.5

Nocturnal anchor TIB (hr)	**4.2**	**5.2**	**4.2**	**6.2**	**5.2**	**6.2**	**5.2**	**6.2**	**8.2**
Diurnal nap TIB (hr)	2.0	1.2	2.4	0.4	1.6	0.8	2.0	1.2	0
Total TIB (h) per 24 hr	6.2	6.4	6.6	6.6	6.8	7.0	7.2	7.4	8.2
Total Sleep Time (min)	L202.9	**	215.1	343.7	**	**	**	**	H404.8
Sleep Efficiency (%)	L80.5	**	85.4	92.4	**	**	**	**	82.3
Sleep Latency (min)	H41.3	**	29.0	18.1	**	**	**	**	34.2
% WASO + movement time	6.0	**	5.5	4.7	**	**	**	**	H15.1
% NREM sleep	63.7	**	69.2	70.7	**	**	**	**	65.1
% Stage 1 sleep	3.2	**	2.4	3.9	**	**	**	**	H 5.6
% Stage 2 sleep	39.4	**	46.3	H50.6	**	**	**	**	49.5
% Slow wave sleep (SWS)	21.1	**	20.5	16.2	**	**	**	**	L10.0
% REM sleep	L15.5	**	15.8	21.0	**	**	**	**	17.7

* The condition with the highest mean value and the condition with the lowest mean value for each variable are indicated: Hhighest value(s) among the 10 conditions, scored as of 09-29-00; Llowest value(s) among the 10 conditions, scored as of 09-29-00.

** Conditions where polysomnographic scoring of the data has not been completed as of 09-29-00.

Table 3. Average diurnal nap sleep time per 24 hr. on sleep restriction days. Conditions ordered by total time in bed per 24hr, from shortest to longest.

Nocturnal anchor TIB (hr)	4.2	4.2	4.2	5.2	4.2	5.2	4.2	5.2	6.2
Diurnal nap TIB (hr)	**0**	**0.4**	**0.8**	**0**	**1.2**	**0.4**	**1.6**	**0.8**	**0**
Total TIB (h) per 24 hr	4.2	4.6	5.0	5.2	5.4	5.6	5.8	6.0	6.2
Total Sleep Time (min)	NN	19.4	**	NN	64.8	[L]15.2	**	**	NN
Sleep Efficiency (%)	NN	80.8	**	NN	90.0	[L]63.3	**	**	NN
Sleep Latency (min)	NN	[L]4.3	**	NN	7.2	9.8	**	**	NN
% WASO + movement time	NN	[H]7.3	**	NN	4.2	4.0	**	**	NN
% NREM sleep	NN	62.3	**	NN	[H]70.6	[L]38.1	**	**	NN
% Stage 1 sleep	NN	12.8	**	NN	7.3	11.1	**	**	NN
% Stage 2 sleep	NN	42.3	**	NN	27.3	[L]25.3	**	**	NN
% Slow wave sleep (SWS)	NN	7.2	**	NN	[H]36.0	1.7	**	**	NN
% REM sleep	NN	10.8	**	NN	14.8	10.7	**	**	NN

Nocturnal anchor TIB (hr)	4.2	5.2	4.2	6.2	5.2	6.2	5.2	6.2	8.2
Diurnal nap TIB (hr)	**2.0**	**1.2**	**2.4**	**0.4**	**1.6**	**0.8**	**2.0**	**1.2**	**0**
Total TIB (h) per 24 hr	6.2	6.4	6.6	6.6	6.8	7.0	7.2	7.4	8.2
Total Sleep Time (min)	109.9	**	[H]132.8	[L]15.2	**	**	**	**	NN
Sleep Efficiency (%)	91.6	**	[H] 92.2	[L]63.3	**	**	**	**	NN
Sleep Latency (min)	[H]11.4	**	5.1	8.5	**	**	**	**	NN
% WASO + movement time	3.8	**	6.6	[L]1.6	**	**	**	**	NN
% NREM sleep	58.0	**	69.6	57.4	**	**	**	**	NN
% Stage 1 sleep	5.1	**	[L]4.3	[H]16.1	**	**	**	**	NN
% Stage 2 sleep	31.3	**	[H]42.7	39.7	**	**	**	**	NN
% Slow wave sleep (SWS)	21.6	**	22.6	[L]1.6	**	**	**	**	NN
% REM sleep	[H]26.4	**	20.9	[L]3.1	**	**	**	**	NN

* The condition with the highest mean value and the condition with the lowest mean value for each variable are indicated: [H]highest value(s) among the 10 conditions, scored as of 09-29-00; [L]lowest value(s) among the 10 conditions, scored as of 09-29-00.

** Conditions where polysomnographic scoring of the data has not been completed as of 09-29-00.

NN Conditions where there was no diurnal nap (i.e. diurnal nap TIB = 0)

4. **Explore hormone secretions in relation to anchor and nap sleep durations (specific aim 3).**

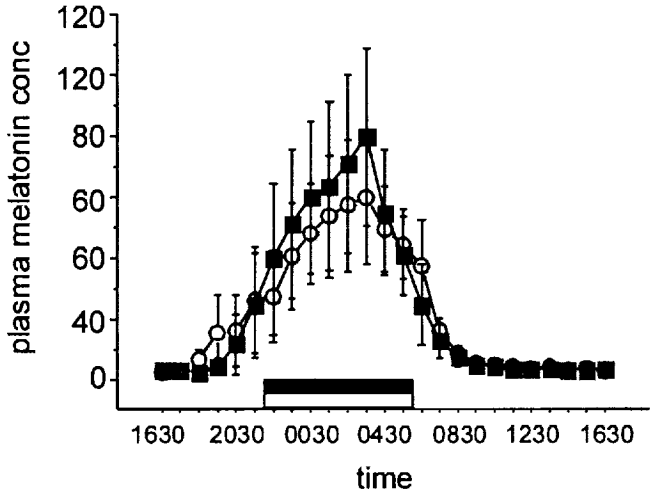
Blood samples were collected from a venous catheter at 15 minutes intervals for a period of 25 hours (1630hr to 1730h the next day) on day 1 (baseline day 1) and day 12 (sleep restriction days 9 through 10). Blood samples collected at 60-minute intervals were assayed for the presence of melatonin and cortisol (1630h, 1730h, 1830h etc. for melatonin; 1645h, 1745h, 1845h etc. for cortsiol). Blood samples collected at 15-minute intervals (1630h, 1645h, 1700h etc.) were assayed for the presence of growth hormone. During the blood collection periods subjects were maintained in a quasi-constant routine paradigm, remaining in a semi-supine position in bed, with the timing of meals held constant on both days. Subjects were required to sleep at the appropriate times (i.e. either baseline or condition). The blood samples were collected using a novel non-thrombogenic catheter and blood pump device that allowed rapid sampling (15-min. intervals) for an extended period of time. This innovative design allowed for us to collect blood samples for a 25-hour duration, without having to flush the vein with heparin, and thereby reducing burden on the subject. In order to determine the effect of the sleep restriction profile on the circadian profile of these hormones, data are being analyzed using Lomb-Scargle analysis to determine the presence of circadian (or other) rhythmicity. In addition, deconvolution techniques are being used to calculate the total amount of hormone secreted under baseline conditions (i.e., 8hr time in bed) and sleep restriction conditions (restriction days 9-10).

a. Melatonin secretory profiles (specific aim 3). Analysis of the effect of chronic sleep restriction with and without naps on the secretion of melatonin is underway. Since there did not appear to be a significant effect of varying nap duration on melatonin secretion, the melatonin data have been pooled according to anchor sleep duration. As can be seen in Figure 5, and as expected, there was no significant difference in melatonin secretion on baseline day 1 and sleep restriction day 10 relative to day 1 for the 8.2hr control condition, which involved 8.2 hr time in bed on both days (graph A). However, when anchor sleep duration was shortened on the 10th day of restriction to either 5.2hr (graph C) or 4.2hr (graph D), a phase delay was evident in both the onset and offset of melatonin secretion. It is unlikely that the changes in melatonin profile under more severe nocturnal sleep restriction is due to direct, inhibitory effects of light exposure (< 50 lux ambient) on melatonin secretion. If this were occurring, we would expect to observe a shortening of the period of melatonin secretion, since in the sleep restriction conditions sleep was truncated, and hence light administration increased, both prior to and following the timing of sleep relative to the 8.2hr baseline sleep period. We believe this effect to be mediated via the circadian system. In order to further investigate this hypothesis, we are analyzing core body temperature data (rectal probe), since this measure affords an additional marker of circadian phase. Not surprisingly, additional analyses of melatonin data have demonstrated a significant circadian rhythm in melatonin secretion present on both baseline and sleep-restriction days. Although we were able to collect over 80% of the blood samples in two thirds of the subjects, some of the subjects had insufficient data to complete the circadian analysis (this was due to difficulties with blood draws and hemolyzation). Visual inspection of the graphs, however, indicated that in these subjects a typical secretory pattern for melatonin (i.e. circadian) was present. Analyses on these and additional melatonin data continue.

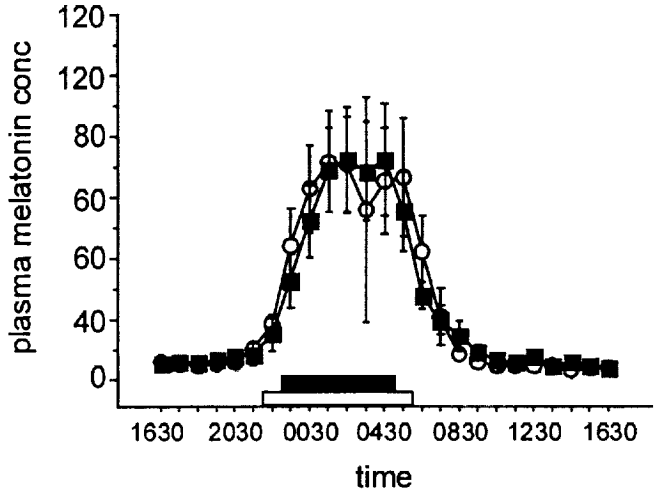
b. Cortisol secretory profiles (specific aim 3). Analysis of the effect of chronic sleep restriction on cortisol secretory profiles is still underway. Presented below in Figure 6 is the effect of chronic sleep restriction (graph B) on cortsiol relative to the control sleep (graph A). The expected circadian peak in cortsiol secretion following sleep offset is evident on both nights in the 8.2hr control condition (graph A), and on the baseline day (8.2hr) in the restriction condition (graph B). After restricting nocturnal anchor sleep to 4.2hr for 10 days, the circadian profile in cortisol remained, but there was an earlier peak in cortisol at awakening from the 4.2hr sleep. This phase advanced cortisol peak appears to reflect the advanced waking time of the restricted sleep condition. We are conducting additional analyses to determine whether it is associated with increased stress from being forced to awaken after reduced nocturnal sleep, or whether it is an advance in the cortisol rhythm (which if it were the case would be opposite of what was seen for melatonin under 4.2hr sleep restriction (Figure 5D).

Figure 5: Plasma melatonin profiles (mean and sem) on baseline day 1 (8hr time in bed; open circles and open abscissa bar) and sleep restriction day 10 (closed squares and closed abscissa bar) for four representative conditions: A. control condition 8.2hr time in bed on day 1 and day 10 (2154hr to 0606hr); B; 8.2hr time in bed on day 1 and 6.2hr time in bed on restriction day 10 (2254hr to 0506hr); C. 8.2hr time in bed on day 1 and 5.2hr time in bed on restriction day 10 (2324hr to 0436hr); and D. 8.2hr time in bed on day 1 and 4.2hr time in bed on restriction day 10 (2354hr to 0406hr). Blood samples for melatonin analysis were collected at 60-min. intervals in ambient light levels below 50 lux. There is a phase delay apparent in the data produced by restricting nocturnal anchor sleep to 5.2hr (graph C) and 4.2hr (graph D).

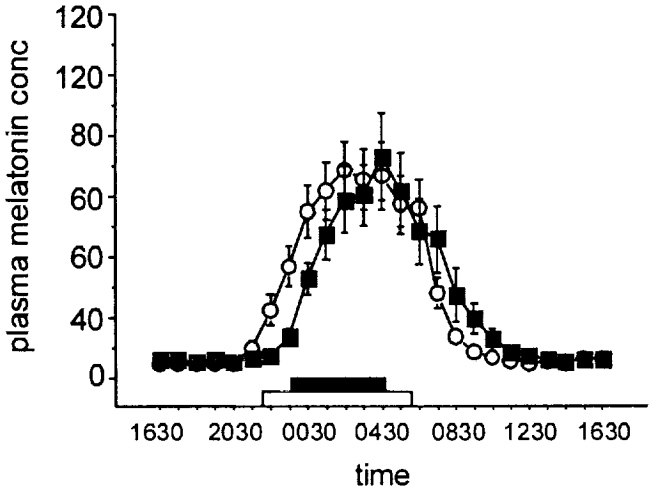
A. 8.2 hour anchor condition (N=5)



B. 6.2 hour anchor condition (N=3)



C. 5.2 hour anchor condition (N=12)



D. 4.2 hour anchor condition (N=12)

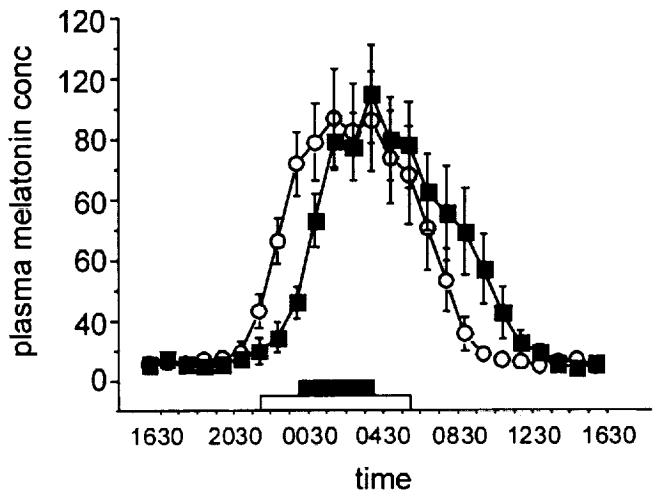


Figure 6. Plasma cortisol profiles (mean and sem) on baseline day 1 (8hr time in bed; open abscissa bar) and sleep restriction day 10 (closed abscissa bar) for two conditions: A. control condition 8.2hr time in bed on day 1 and day 10 (2154hr to 0606hr); and B; 8.2hr time in bed on day 1 and 4.2hr time in bed on restriction day 10 (2354hr to 0406hr). In graph A, open circles represent data from the baseline day and closed boxes represent the data from restriction day 10 (8.2hr). In graph B, however, these symbol representations were inadvertently reversed—open circles represent data from sleep restriction day 10 (4.2hr), while closed boxes represent the data from the baseline day (8.2hr). Blood samples for cortisol analysis were collected at 60-min. intervals.

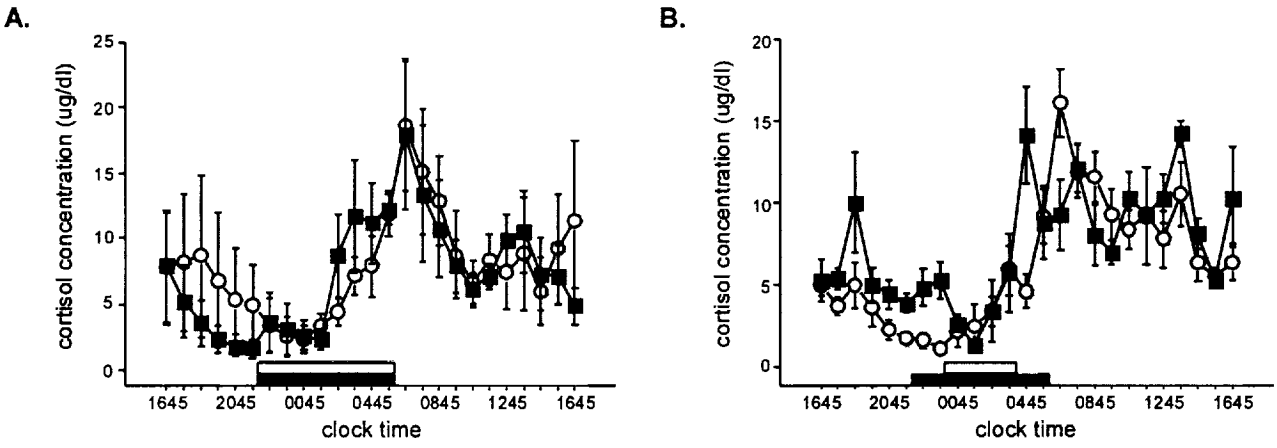
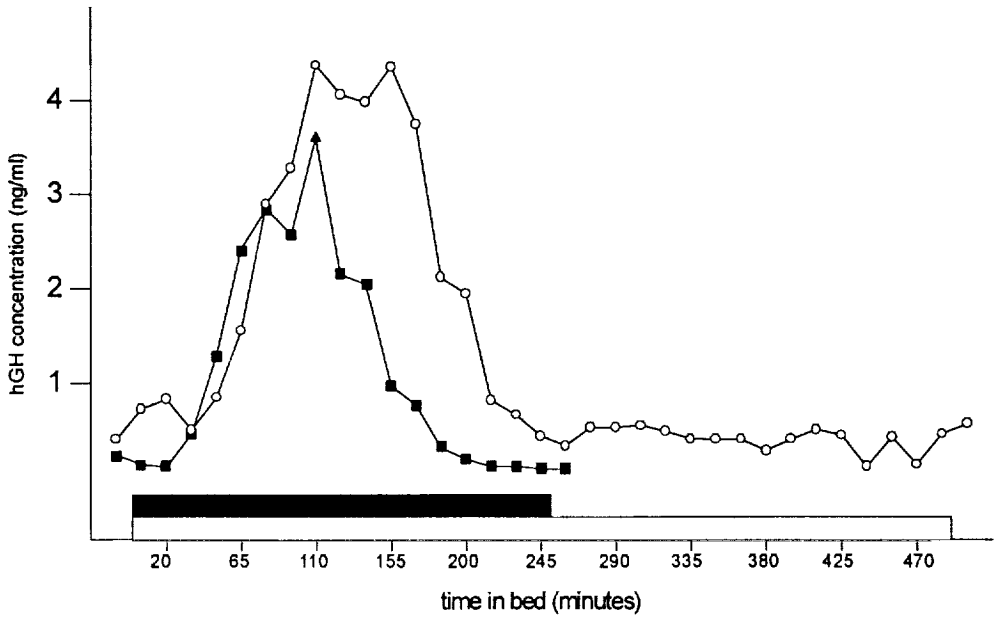


Figure 7. Plasma growth hormone profiles on baseline day 1 (8hr time in bed; open abscissa bar and open circles) and sleep restriction day 10 (closed abscissa bar and closed squares) for the 4.2hr time in bed nocturnal anchor sleep condition. Blood samples for hGH analysis were collected at 15-min. intervals from 1630hr to 1730hr the following day.



c. Growth hormone secretory profiles (specific aim 3). Analysis of the effect of chronic sleep restriction with and without naps on the secretion of growth hormone is being completed. Presented below in Figure 7 is the effect of a chronic reduction in nocturnal anchor sleep to 4.2 hours time in bed per night on growth hormone secretion. Growth hormone secretion occurs in a pulsatile fashion across the twenty-four hour day, with approximately 70% of hGH being secreted during sleep. In contrast to other nocturnally secreted hormones such as melatonin, which are primarily controlled by the circadian system, with secretion persisting in the absence of sleep, hGH is primarily influenced by the sleep-wake system, as well as by food intake, activity level and gender (with females secreting high levels than males). During total sleep deprivation, the secretion of hGH is significantly reduced. Consistent with this, in the present study when sleep was reduced to 4.2hr per night there was a significant reduction in hGH secretion relative to when 8.2hr of sleep was obtained. Planned analyses are underway to determine whether the amount of hGH secretion varied across the 18 anchor + nap sleep conditions according to key features of sleep physiology, especially the timing and amount of slow wave sleep. The results of these analyses will provide needed information on the extent to which specific restricted sleep schedules promote normal hGH secretion levels as well as optimal neurobehavioral functioning.

APPENDIX B

Papers, reviews, book chapters, abstracts published or submitted for publication and supported in whole or in part by the NSBRI

Total N = 31 from October 1, 1997 to September 30, 2000

Papers (n = 9):

Dinges, DF, Achermann, P: Future considerations for models of human neurobehavioral function. Journal of Biological Rhythms, 14(6):598-601, 1999.

Jewett, ME, Dijk, D-J, Kronauer, RE, Dinges, DF: Dose-response relationship between sleep duration and human psychomotor vigilance and subjective alertness. Sleep, 22:171-179, 1999.

Van Dongen, HPA, Juijpers, MGCE, Duindam, H, Kerkhof, GA: A case study of the free-running circadian period of a morning type subject in time isolation. Sleep-Wake Research in The Netherlands 10:113-118, 1999.

Van Dongen, HPA, Olofsen, E, VanHartevelt, JH, Kruyt, EW: A procedure of multiple period searching in unequally spaced time-series with the Lomb-Scargle method. Biological Rhythm Research 30(2):149-177, 1999.

Van Dongen, HPA, Olofsen, E, VanHartevelt, JH, Kruyt, EW: Searching for biological rhythms: Peak detection in the periodogram of unequally spaced data, Journal of Biological Rhythms 14(6):617-620, 1999.

Doran, SM, Van Dongen, HPA, Dinges, DF: Sustained attention performance during sleep deprivation: Evidence of state instability. Archives of Italian Biology, in press.

Shearer, WT, Reuben, JM, Mullington, JM, Price, NJ, Lee, BN, Smith, EO, Szuba, MP, Van Dongen, HPA, Dinges, DF: Soluble tumor necrosis factor-alpha receptor 1 and interleukin-6 plasma levels in humans subjected to the sleep deprivation model of space flight. Journal of Allergy and Clinical Immunology, in press.

Van Dongen, HPA, Kerkhof, GA: Repeated assessment of the endogenous 24-hour profile of blood pressure under constant routine. Chronobiology International, in press.

Meier-Ewert, HK, Ridker, PM, Rifai, N, Price, N, Dinges, DF, Mullington, JM: Absence of diurnal variation of c-reactive protein levels in healthy human subjects. Clinical Chemistry, submitted.

Chapters (n = 3):

Van Dongen, HPA, Dinges, DF: Circadian rhythms in fatigue, alertness and performance. In: M.H. Kryger, T. Roth, & W.C. Dement (Eds.), Principles and practice of sleep medicine (3rd edition). Philadelphia, PA: W.B. Saunders, pp. 391-399, 1999.







Chugh, DK, Dinges, DF: Mechanisms of sleepiness. In: A.I. Pack (Ed.), Pathogenesis, Diagnosis, and Treatment of Sleep Apnea. New York: Marcel Dekker, Inc., in press.

Kloss, JD, Szuba, MP, Dinges, DF: Sleep loss and sleepiness: Physiological and neurobehavioral effects. In: Neuropsychopharmacology: The Fifth Generation of Progress, Baltimore: Lippincott, Williams and Wilkins, in press.

Published abstracts (n = 19)

Dinges, DF, Czeisler, CA: Summary of sleep/circadian rhythm technical session. Proceedings of the First Biennial Space Biomedical Investigators' Workshop, League City, TX, pp. 534-537, 1999.

Dinges, DF, Maislin, G, Kuo, A, Carlin, MM, Powell, JW, Van Dongen, H, Mullington, J: Chronic sleep restriction: Neurobehavioral effects of 4hr, 6hr, and 8hr TIB. Sleep, 22 (Supplement):S115 (abstract), 1999.

Dinges, DF, Shearer, WT, Reuben, JM, Mullington, JM, Price, N, Szuba, MP, Kapoor, S, Van Dongen, HPA: Human immune status during prolonged sleep deprivation: Circadian rhythmicity, sleep drive, stress, and caffeine. Sleep Research Online 2 (Supplement 1):659 (abstract), 1999.

Dinges, DF, Van Dongen, HPA: Countermeasures to neurobehavioral deficits from cumulative partial sleep deprivation during space flight. Proceedings of the First Biennial Space Biomedical Investigators' Workshop, League City, TX, pp. 551-553, 1999.

Konowal, NM, Van Dongen, HPA, Powell, JW, Mallis, MM, Dinges, DF: Determinants of microsleeps during experimental sleep deprivation. Sleep, 22 (Supplement):S328-S329 (abstract), 1999.

Mallis, MM, Maislin, G, Powell, JW, Konowal, NM, Dinges, DF: Perclos predicts both PVT lapse frequency and cumulative lapse duration. Sleep 22, (Supplement):S149, (abstract), 1999.

Mullington, JM, Carlin, MM, Kapoor, SC, Samuel, S, Price, N, Szuba, MP, Dinges, DF: Sleep deprivation elevates the circadian nadir in cortisol secretion. Sleep, (Supplement): S329 (abstract), 1999.

Neri, DF, Mallis, MM, Oyung, RL, Dinges, DF: Do activity breaks reduce sleepiness in pilots during a night flight? Sleep, 22 (Supplement):S150 (abstract), 1999.

Van Dongen, HPA, Mailsin, G, Dinges, DF: Chronic partial sleep deprivation: Neurobehavioral response and sleep architecture. Sleep Research Online 2 (Supplement):S735 (abstract), 1999.

Van Dongen, HPA, Olofsen, E, VanHartevelt, JH, Kruyt, EW: Power spectral analysis: Generalizing the invariance to time translation. Workshop on Biomathematical Models of Circadian Rhythmicity, Sleep Regulation and Neurobehavioral Function in Humans, Dedham, Massachusetts: *3*, (abstract), 1999.

Van Dongen, HPA, Powell, JW, Konowal, NM, Brodnyan, CG, Mullington, JM, Dinges, DF: The dynamics of chronically restricted sleep and recovery sleep. Sleep, *22* (Supplement):S116-S117 (abstract), 1999.

Andersen, CM, Maislin, G, Van Dongen, HPA, Rogers, NL, Powell, JW, Carlin, MM, Mullington, JM, Dinges, DF: Effect of chronically reduced nocturnal sleep, with and without daytime naps, on neurobehavioral performance. Sleep 23 (Supplement 2): A74-A75 (abstract), 2000.

Dinges, DF, Doran, SM, Mullington, J, Van Dongen, HP, Price, N, Samuel, S, Carlin, MM, Powell, JW, Mallis, MM, Martino, M, Brodnyan, C, Konowal, N, Szuba, MP: Neurobehavioral effects of 66 hr of sustained low-dose caffeine during 88 hr of total sleep deprivation. Sleep 23 (Supplement 2): A20-A21 (abstract), 2000.

Ecker, AJJ, Schaechter, J, Price, N, Rogers, NL, Mullington, JM, Szuba, MP, Van Dongen, HPA, Dinges, DF: Changes in plasma melatonin secretion following chronic sleep restriction. Sleep 23 (Supplement 2): A184-A185 (abstract), 2000.

McManus, KS, Szuba, MP, Carlin, MM, Orne, EC, Dinges, DF: Total sleep deprivation induces opposite effects on mood states in depressed and control subjects. Sleep 23 (Supplement 2): A37 (abstract), 2000.

Mullington, JM, Mantzoros, CS, Samaras, J, Price, N, Samuel, S, Carlin, M, Szuba, M, Dinges, DF: Circadian rhythm amplitude of leptin is reduced by chronic sleep restriction to 4 hours per night. Sleep 23 (Supplement 1): A71 (abstract), 2000.

Rogers, NL, McMahon, J, Lushington, K, Dawson, D: Effect of knowledge of bedtime on sleep onset and body temperature. Sleep 23 (Supplement 2): A56 (abstract), 2000.

Rogers, NL, Price, N, Mullington, JM, Szuba, MP, Van Dongen, HPA, Dinges, DF: Plasma cortisol changes following chronic sleep restriction. Sleep 23 (Supplement 2): A70-A71 (abstract), 2000.

Van Dongen, HPA, Doran, SM, Dinges, DF: Performance during sleep deprivation: Evidence for state instability and trait vulnerability. Sleep 23 (Supplement 2): A245-A246 (abstract), 2000.

APPENDIX C

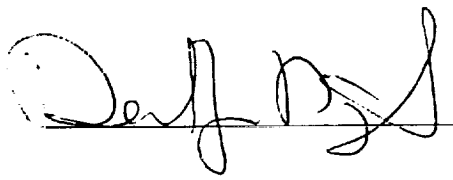
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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Report

Research Team:	**Human Performance Factors, Sleep and Chronobiology Team**
Project Name:	**Quantitative EEG Monitoring of Vigilance: Effect of Sleep Deprivation, Circadian Phase and Sympathetic Activation**
Principal Investigator:	**Derk-Jan Dijk, Ph.D.** **Reader in Physiology** **Centre for Chronobiology** **School of Biomedical and Life Sciences** **University of Surrey** **Guildford GU2 7XH United Kingdom** **Telephone: + 44 1483 879341; Telefax +44 870 1371590;** **E-mail d.j.dijk@surrey.ac.uk**
Project Investigator:	**Christian Cajochen, Ph.D.** **Instructor in Medicine** **Circadian, Neuroendocrine and Sleep Disorders Section** **Brigham and Women's Hospital, Harvard Medical School** **Steven W. Lockley, Ph.D.** **Lecturer in Biochemistry** **Centre for Chronobiology** **School of Biomedical and Life Sciences** **University of Surrey**



Date 30 Jan 00

EXECUTIVE SUMMARY

Shuttle astronauts typically sleep only 6 to 6.5 hours per day while in orbit. This sleep loss is related to recurrent sleep cycle shifting--due to mission-dependent orbital mechanics and mission duration requirements-- and associated circadian displacement of sleep, the operational demands of space flight, noise and space motion sickness. Such sleep schedules are known to produce poor subjective sleep quality, daytime sleepiness, reduced attention, negative mood, slower reaction times, and impaired daytime alertness. Countermeasures to allow crew members to obtain an adequate amount of sleep and maintain adequate levels of neurobehavioral performance are being developed and investigated. However, it is necessary to develop methods that allow effective and attainable in-flight monitoring of vigilance to evaluate the effectiveness of these countermeasures and to detect and predict online critical decrements in alertness/performance. There is growing evidence to indicate that sleep loss and associated decrements in neurobehavioral function are reflected in the spectral composition of the electroencephalogram (EEG) during wakefulness as well as in the incidence of slow eye movements recorded by the electro-oculogram (EOG). Furthermore, our preliminary data indicated that these changes in the EEG during wakefulness are more pronounced when subjects are in a supine posture, which mimicks some of the physiologic effects of microgravity. Therefore, we evaluated the following hypotheses: (1) that during a 40-h period of wakefulness (i.e., one night of total sleep deprivation) neurobehavioral function deteriorates, the incidence of slow eye-movements and EEG power density in the theta frequencies increases especially in frontal areas of the brain; (2) that the sleep deprivation induced deterioration of neurobehavioral function and changes in the incidence of slow eye movements and the spectral composition of the EEG are more pronounced when subjects are in a supine position; and (3) that based on assessment of slow-eye movements and quantitative on-line topographical analyses of EEG during wakefulness an EEG and or EOG parameter can be derived/constructed which accurately predicts changes in neurobehavioral function.

In a series of experiments and data analysis projects conducted during the first three years of this project we have established that:

1. The spectral composition of the EEG during wakefulness exhibits pronounced and predictable changes during a 24-h period of sustained wakefulness.
2. The changes associated with sleep loss are most pronounced in EEGs derived from frontal areas of the brain, and in particular so in the delta and theta frequencies, both during wakefulness and during sleep.
3. Changes in alertness and psychomotor vigilance correlate with changes in EEG power density in the delta and theta frequencies in frontal derivations.
4. The incidence of slow eye movements during wakefulness increases during sleep loss and correlates with changes in alertness and psychomotor vigilance. This correlation is so tight that inter-individual differences in the time course of the incidence of slow eye movements closely resemble the inter-individual differences in the time course of neurobehavioral performance during a 24-h episode of sustained wakefulness.
5. The circadian pacemaker modulates the incidence of slow eye movements as well as the spectral composition of the EEG during wakefulness.
6. Light-induced changes in the amplitude of the circadian pacemaker and associated changes in the amplitude of the circadian modulation of alertness are associated with

changes in the amplitude of the circadian modulation of the incidence of slow eye movements

7. Light-induced acute changes in alertness are associated with acute changes in the EEG as well as with the incidence of slow eye movements during wakefulness.
8. Posture modulates the apparent amplitude of the circadian rhythm of body temperature and heart rate such that this amplitude is reduced when subjects are in a supine posture during 40-h of wakefulness.
9. Posture modulates the effects of sleep loss and circadian phase on neurobehavioral performance as assessed by the psychomotor vigilance test such that the detrimental effects of sleep loss/circadian phase are more pronounced when subjects are in a supine posture during 40-h of wakefulness.
10. The incidence of slow-eye movements during a drowsiness test predicts performance on a psychomotor vigilance task conducted one hour later.

These data, generated by the hypotheses described above as well as by secondary hypotheses, establish that the original hypotheses, specific aims and their modifications as described in the original proposal and subsequent progress reports, were fruitful. These new findings establish a close and robust association of frontal EEG and ocular parameters with changes in neurobehavioral performance in a variety of protocols in which sleep homeostasis and circadian rhythmicity were manipulated. Circadian rhythmicity and sleep homeostasis have been established to be major determinants of performance and our data establish that they are also major determinants of the waking EEG and ocular parameters. This implies that these parameters are likely to be associated with performance in a variety of conditions in which performance is jeopardized by changes in the status of the sleep homeostat or changes in circadian phase.

Furthermore, these data indicate that EEG/EOG based on-line monitoring of alertness/performance can serve as a practical and attainable tool to predict and prevent critical decrements in performance and alertness, without the need to conduct time consuming tests of neurobehavioral performance.

The research conducted in the current grant period aimed at the development of countermeasures for Human performance failure because of sleep and circadian rhythm problems [Risk 19, Critical Road Map http://criticalpath.jsc.nasa.gov]. In particular, our research relates to the critical questions 6.08 (What are the best methods for monitoring the status of sleep and circadian functioning and for assessing the effects of sleep loss and circadian dysrhythmia that are also portable and non-intrusive in the spaceflight environment?) and 6.21 (What mathematical and experimental models best predict performance problems associated with sleep-wake and work history and circadian rhythm status, and also provide guidelines for successful countermeasure strategies?). In addition, our research is relevant to critical question 6.05 (What are the acute and long-term effects of exposure to the space environment on biological rhythmicity, on sleep architecture, quality, and quantity, and their relationship to performance capability?) and 6.06 (Which countermeasure or combination of behavioral and physiological countermeasures will optimally mitigate specific performance problems associated with sleep loss and circadian disturbances during a Mars mission?).

Further understanding of the relationship between EEG/EOG and neurobehavioral function could thus have a profound effect on the health, productivity and safety of astronauts during space missions.

The research is relevant for the round-the-clock work schedules (day, evening and

night work) on the International Space Station, the altered sleep/wake schedule on a Mars surface station, or any other situation where the work-rest schedule is shifted and sleep loss is incurred. It also has relevance for ground personnel monitoring orbiting crew members who must do so working round-the-clock schedules.

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I PROJECT RESEARCH ACTIVITY

A. HYPOTHESES, OBJECTIVES & SPECIFIC AIMS FROM ORIGINAL PROPOSAL

The success and effectiveness of space flights depend on the ability of crew members to maintain a high level of cognitive performance and vigilance while operating and monitoring sophisticated instrumentation. Shuttle astronauts typically lose 2 hours of sleep per day while in orbit. This sleep loss is related to recurrent sleep cycle shifting due to mission-dependent orbital mechanics and mission duration requirements, the operational demands of space flight, noise and space motion sickness. Such cumulative sleep loss is known to produce predictable changes in sleep structure and the spectral composition of the EEG during sleep, daytime sleepiness, reduced attention, negative mood, slower reaction times, gastrointestinal disorders and impaired daytime alertness. These decrements in performance, sleep and well-being will minimally lead to reduced efficiency of task performance, increased discomfort and increased errors of the crew members, which could compromise their safety and health. The need to develop effective but safe countermeasures to allow crew members to obtain an adequate amount of sleep has been recognized and such countermeasures are being investigated. However, it is necessary to develop methods which allow effective and attainable in-flight monitoring of vigilance to evaluate the effectiveness of these countermeasures and to online detect and predict critical decrements in alertness/performance. During the past decade we and others have demonstrated that the spectral composition of the EEG during nonREM sleep accurately reflects the status of the sleep homeostat, i.e. there is a quantitative relationship between EEG power density and sleep loss even within the physiologic range of 1 to 2 hours of sleep loss. There is growing evidence to indicate that the status of the sleep homeostat and the effects of sleep loss and decrements in neurobehavioral function are also reflected in the spectral composition of the EEG and characteristics of the EOG during wakefulness. Furthermore, our preliminary data indicate that these changes in the EEG during wakefulness are more pronounced when subject are in a supine posture i.e. when sympathetic activity is reduced, suggesting that in microgravity similar changes may occur. Therefore, we propose to evaluate the following specific aims:

(1) To test the hypothesis that during a 40-h period of wakefulness (i.e. one night of total sleep deprivation) neurobehavioral function deteriorates and EEG power density in the theta frequencies increases especially in frontal areas of the brain.

(2) To test the hypothesis that the sleep deprivation induced deterioration of neurobehavioral function and changes in the spectral composition of the EEG are more pronounced when subjects are in a supine position.

(3) To test the hypothesis that based on quantitative on-line topographical analyses of EEG during wakefulness an EEG parameter can be derived/constructed which accurately predicts changes in neurobehavioral function.

B. MODIFICATIONS REQUIRED & RATIONALE FOR MODIFICATIONS

In the initial review it was suggested that ocular parameters such as slow eye movements and eye blinks could also be used as indicators of neurobehavioral performance capability and decrements thereof. We therefore have added the assessment of slow-eye movements and eye-blinks to the variables that are quantified during our experiments. The

hypotheses have been modified to reflect this change and now read:

(1) that during a 40 h period of wakefulness (i.e., one night of total sleep deprivation) neurobehavioral function deteriorates, the incidence of slow eye-movements and EEG power density in the theta frequencies increases especially in frontal areas of the brain.

(2) that the sleep deprivation induced deterioration of neurobehavioral function and changes in the incidence of slow eye movements and the spectral composition of the EEG are more pronounced when subjects are in a supine position.

(3) that based on assessment of slow-eye movements and quantitative on-line topographical analyses of EEG during wakefulness an EEG and or EOG parameter can be derived/constructed which accurately predicts changes in neurobehavioral function.

In addition we have collected and analyzed EEG/EOG data in sleep-deprivation and circadian experiments conducted in the Laboratory for Circadian and Sleep Disorders Medicine (Director Charles A. Czeisler, Ph.D., M.D.).

C. SUMMARY OF PROGRESS

During year the first three years of this project we have established that:

1. *The spectral composition of the EEG during wakefulness exhibits pronounced and predictable changes during a 24-h period of sustained wakefulness.* These data a direclty relevant to our first hypothesis and demonstrate that the EEG during wakefulness is responsive to the duration of wakefulness and or circadian phase.
2. *The changes associated with sleep loss are most pronounced in EEGs derived from frontal areas of the brain, and in particular so in the delta and theta frequencies, both during wakefulness and during sleep.* These data, obtained in two experiments, are directly relevant to our hypotheses and may indicate that frontal areas of the brain are more affected by sleep loss. The data also imply that EEG based monitoring of alertness and performance capability is best achieved by monitoring frontal areas of the brain. This has implications for the development of technologies for online monitoring.
3. *Changes in alertness and psychomotor vigilance correlate with changes in EEG power density in the delta and theta frequencies in frontal derivations.* These observations demonstrate an association between EEG and performance. This is a pre-requisite for the use of EEG for monitioring of alerntness/performance.
4. *The incidence of slow eye movements during wakefulness increases during sleep loss and correlates with changes in alertness and psychomotor vigilance. This correlation is so tight that inter-individual differences in the time course of the incidence of slow eye movements closely resemble the inter-individual differences in the time course of neurobehavioral performance during a 24-h episode of sustained wakefulness.* This surprising observation holds promise for the use of ocular parameters for monitoring of vigilance and performance.
5. *The circadian pacemaker modulates the incidence of slow eye movements as well as the spectral composition of the EEG during wakefulness.* Sleep homeostasis and circadian rhythmicity are major contributors to performance. Our data now show that the circadian pacemaker also modulates the incidence of slow eye movements. This gives credence to the use of ocular parameters for monitoring alertness.

6. *Light-induced changes in the amplitude of the circadian pacemaker and associated changes in the amplitude of the circadian modulation of alertness are associated with changes in the amplitude of the circadian modulation of the incidence of slow eye movements* The association between EOG and alertness is preserved when alertness is manipulated by changing the amplitude of the circadian pacemaker.
7. *Light-induced acute changes in alertness are associated with acute changes in the EEG as well as with the incidence of slow eye movements during wakefulness.* The discovery that variations in light levels within the range commonly found in living environments has a profound impact on alertness is relevant for the space environment because on spacecraft light levels can be low. Furthermore, these data demonstrate that the association between alertness and EEG/EOG persists when alertness is manipulated by light exposure.
8. *Posture modulates the apparent amplitude of the circadian rhythm of body temperature and heart rate such that this amplitude is reduced when subjects are in a supine posture during 40-h of wakefulness.* A supine posture is often used as a model for microgravity. Our data show that under such conditions the apparent amplitude of body temperature and heart rate is reduced also under conditions of sleep loss.
9. *Posture modulates the effects of sleep loss and circadian phase on neurobehavioral performance as assessed by the psychomotor vigilance test such that the detrimental effects of sleep loss/circadian phase are more pronounced when subjects are in a supine posture during 40-h of wakefulness.* These observations are directly relevant to our hypothesis that posture modulates the effects of sleep loss. They may also be relevant to space flight, because the data, under the assumption that a supine posture is a valid model for micro gravity, can be taken to indicate that the effects of sleep loss on neurobehavioral performance will be more severe during space flight.
10. *The incidence of slow-eye movements during a drowsiness test predicts performance on a psychomotor vigilance task conducted one hour later.* This observation is directly relevant to our third hypothesis. These data demonstrate that slow-eye movements, recorded during a standardized situation, yield information on performance capability.

These data were obtained in our primary 12-d research protocol, which was completed by all 12 subjects scheduled, as well as in a number of ancillary protocols, data of which were analyzed in relation to our specific aims.

In the research summarized above, we have addressed our specific aim and hypotheses. The hypotheses were, at least in part, confirmed. The data indicate that EEG and EOG monitoring methods can be developed to assess the status of the sleep homeostat and performance capability and indicate that technologies based on these parameters could be used as countermeasures for the effects of sleep loss and circadian desynchrony such as occurs during space flight.

II IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

Sleep loss, circadian abnormalities and performance decrements have been observed during space flight. The impact of our research on future space biomedical research is primarily related to the development of countermeasures for Human performance failure. We applied several models for performance failure. These models included sleep deprivation, sleep deprivation in combination with a supine posture, desynchrony between the sleep-wake cycle and circadian rhythmicity and altered light levels. In all of these models, performance decrements were observed and in all of these models associations between EEG/EOG and performance or alertness were present. Specifically, the research conducted in the current grant period aimed at the development of countermeasures for Human performance failure because of sleep and circadian rhythm problems [Risk 19, Critical Road Map http://criticalpath.jsc.nasa.gov]. In particular, our research relates to the critical questions 6.08 (What are the best methods for monitoring the status of sleep and circadian functioning and for assessing the effects of sleep loss and circadian dysrhythmia that are also portable and non-intrusive in the spaceflight environment?)

The data imply that methods for monitoring performance can be developed further and that such methods could be portable and non-intrusive, which is a prerequisite for their application during space flight.

Our approach has been to develop these methods in protocols in which two fundamental processes underlying variation in performance are varied. These two processes, sleep homeostasis and circadian rhythmicity, are affected by space flight. We therefore believe that our current methods of monitoring of performance will not only be valid in our ground-based research, but could also be valid during space flight.

To be able to unobtrusively and continuously monitor and predict performance capability can contribute to the success of long duration space flight. The observation that associations between performance decrements and EOG parameters are so tight that they persist at the level of the individual, demonstrates that these methods could complement prediction of performance based on mathematical models, because, so far, these models can only predict group data.

Further development of these methods will require the development of techniques and algorithms to use EEG and EOG information online. In our experiments, this information was used only off line. Theoretically though, there is no reason why this information could not be used online. Development of such methods is necessary before these methods can be implemented.

In our experiments we have used total sleep deprivation as a primary tool to manipulate performance. Total sleep deprivation is a powerful tool to induce performance decrements rapidly. During space flight, astronauts incur chronic partial sleep loss, rather than total sleep loss. The methods applied in our research need to be validated in chronic partial sleep deprivation protocols which are more representative of the conditions during space flight.

Our observation that variations in light levels within the range of room light has a major impact on performance and its electrophysiologic and ocular correlates has major implications for the optimal lighting conditions during space flight. The data demonstrate that light levels which are sufficient to synchronize circadian rhythms when sleep-wake cycles are regular, are not sufficient to maintain optimal performance. This effect of light, which has received little attention, needs to be taken into account when designing the lighting environment in spacecraft.

Our data have also implications for future Earth medical research. Performance decrements

related to sleep loss and circadian phase misalignment are observed in certain shift-work situation. These performance decrements can have fatal consequences. Methods to predict and monitor such decrements could be applied in many settings. Likewise, performance decrements during night shift situations may be prevented or reduced by optimizing the light environment.

Taken together, our research holds promise for the development of countermeasures for neurobehavioral performance decrements during space flight and on Earth.

Appendixes

A. Project Research Data

PRIMARY 12-DAY RESEARCH PROTOCOL:

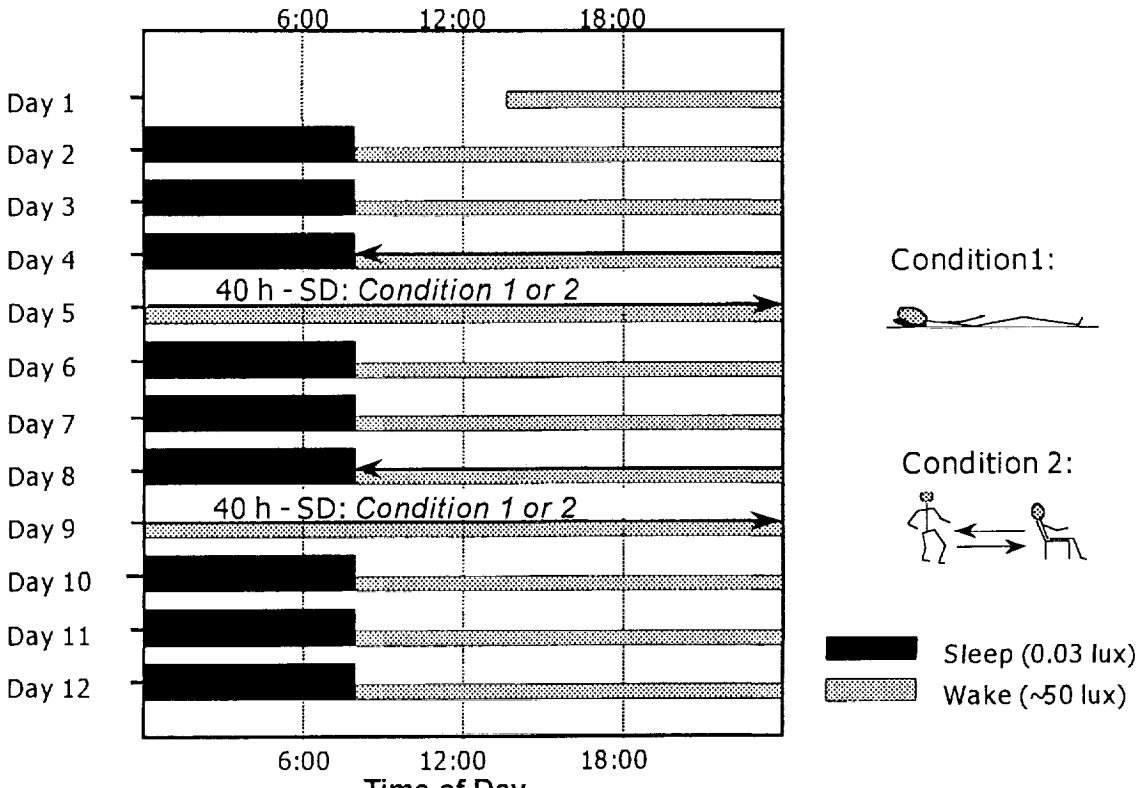
Effect of sleep deprivation and posture on multi-channel EEG, EOG and neurobehavioral performance

Experimental Subjects.
In this protocol 12 subjects were scheduled to be studied during the three year grant period. In year three the remainings subjects were recruited to complete the 12 subjects for this study. Only subjects who have provided written, informed consent for their participation in the study were considered for study. Those volunteers who met all of the screening criteria outlined below were selected for study. All potential subjects were required to maintain a regular sleep/wake schedule for two weeks prior to the start of study.

Experimental Procedure.

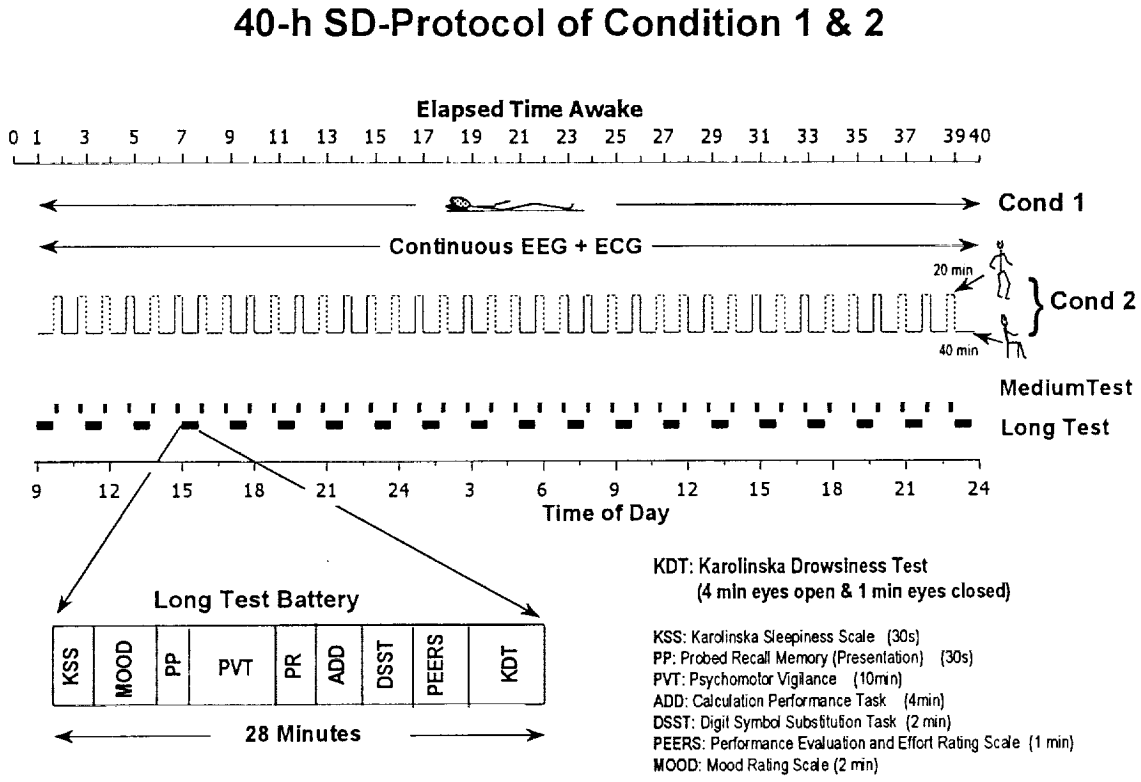
Ambulatory Baseline. The ambulatory baseline segment consists of 14 days, during which wrist activity and light levels will be recorded using ambulatory recording devices while the subjects are at home on a normal routine, maintaining a sleep log.

Baseline. The baseline segment begins with admission to the Intensive Physiologic Monitoring (IPM) Unit of the Brigham and Women's Hospital on Experimental Day 1 as illustrated in the figure and ends on the morning of Experimental Day 4. Physiologic, neurobehavioral and EEG/EOG monitoring (as described in detail in the General Methodology section below) commence upon admission on Experimental Day 1 and will continue throughout the duration of the study. Each day, subjects will be required to perform



a battery of neurobehavioral tests every two waking hours. Subjects will continue to sleep and wake at their regularly scheduled times for three normal scheduled nights (solid black bars). Subject's sleep will be recorded polysomnographically during each Experimental Night.

Sleep Deprivation (40 h). There will be two sleep deprivation episodes. The first 40-h sleep deprivation period will begin on the morning of Experimental day 4 and the second 40-h sleep deprivation will begin on the morning of Experimental day 8. During one sleep deprivation subjects will be in a supine posture while in the other sleep deprivation period subjects will be on a fixed schedule in which they alternate between sitting (40 min) and standing/walking (20 min). The order of these two sleep deprivation conditions will be randomnized and balanced. Blood will be sampled for melatonin starting prior to the sleep



episode preceding the sleep deprivation period and continue until the end of the sleep episode following the sleep deprivation period. The sampling frequency will be three samples per hour. EEG/EOG monitoring will be identical to the Baseline segment.

Recovery. The recovery segment will begin with sleep episode 4 and 7. Neurobehavioral and EEG/EOG monitoring will continue similar to the Baseline and Sleep Deprivation Segment. Subjects will be discharged upon awakening on Experimental Day 13.

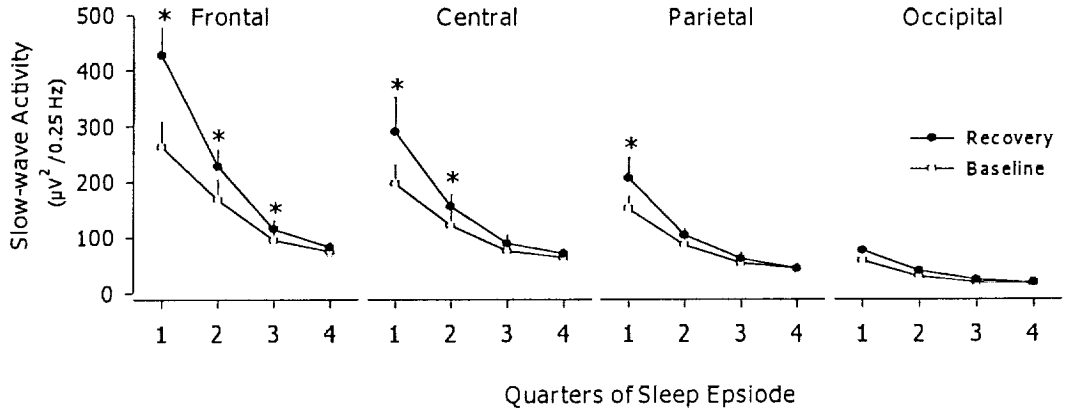
RESEARCH FINDINGS

a. Frontal predominance of EEG slow-wave activity during recovery sleep

We had previously shown that during wakefulness the EEG derived from frontal areas of the brain is more susceptible to the effects of sleep loss than EEGs derived from posterior areas. Although the effects of sleep loss on the sleep EEG have been described, the majority of sleep studies in humans have focused on a single central EEG derivation. However, a

recent study has shown that EEG power density in the delta range (0.75-4.5 Hz) during nonREM sleep is predominant in the frontal region at the beginning of sleep, and that this predominance gradually decreases. To investigate this phenomenon further we hypothesized that the increase in low frequency components of the EEG as induced by sleep deprivation, would be most prominent in frontal derivations.

Following three scheduled days and nights in the laboratory during which subjects slept at their habitual times, four male and two female subjects, age range 19-28 years, underwent a constant routine protocol (CR) which lasted 40h (total sleep deprivation). The EEG was derived from Fz, Cz, Pz, and Oz, referenced against linked mastoids (A1, A2)



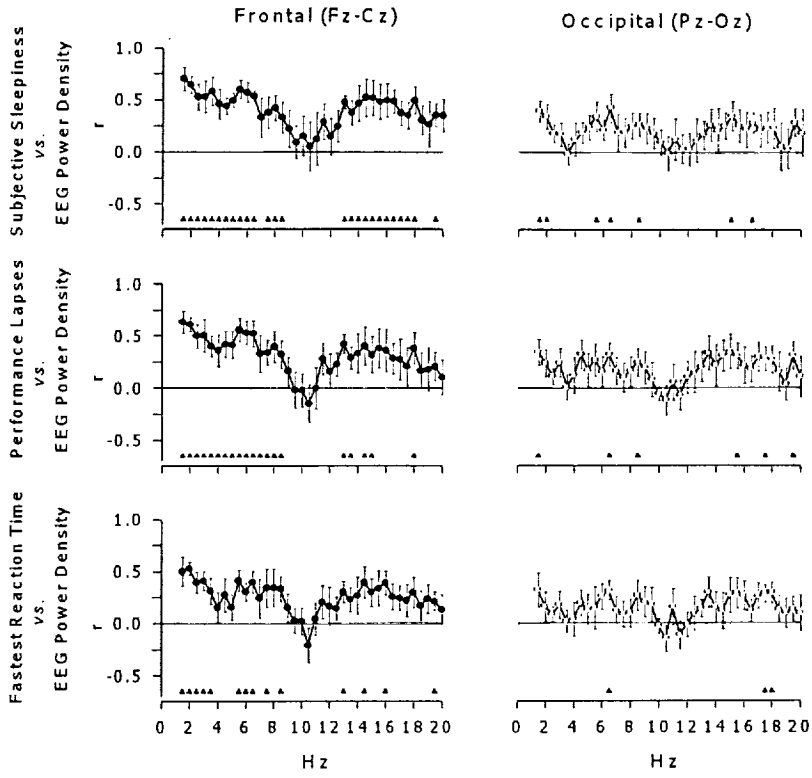
together with two EOGs, one ECG and one EMG. All signals were on-line digitized, and artifact-free 4-s epochs were subjected to a fast Fourier transform routine and then averaged over 20 seconds. The temporal evolution of slow wave activity SWA (EEG power density in the 0.75-4.5 Hz band) was computed for each quarter (2-h interval) of the baseline and recovery sleep episode.

An increase in SWA after sleep deprivation was observed for all locations. A three way rANOVA with factors 'night', 'location' and 'time' revealed a significant main effect for the factor 'night' ($F_{1,5} = 30.5$; $p < 0.003$), 'location' ($F_{3,15} = 22.6$; $p < 0.002$) and 'time'. ($F_{3,15} = 26.3$; $p < 0.002$) and a three-way interaction between the factors ($F_{9,45} = 8.55$; $p < 0.006$). SWA in the frontal derivation was significantly higher in the first, the second and third quarter of the sleep episode. In the central derivation, SWA was higher in the first and second quarter, whereas for the parietal derivation significant higher SWA values were only found in the first quarter (Duncan's multiple range test $p<0.05$).

The data demonstrate that the activation of sleep regulatory processes by an extension of wakefulness from 16 to 40 h results in an increase in low frequency EEG activity that is most pronounced in frontal cortical areas. It therefore seems that sleep loss primarily affects frontal areas of the brain during both sleep and wakefulness.

b. Frequency and EEG derivation specific correlation between EEG and psychomotor vigilance performance and alertness

In an exploratory analyses we investigated whether the association between EEG and neurobehavioral performance is EEG-frequency and EEG-derivation specific. Waking EEG power density and neuro-behavioral performance were assessed in a cohort of ten healthy men during 32 hours of sustained wakefulness. For each subject, waking EEG power density in each 0.5-Hz frequency bin between 1-20 Hz was binned in 2-h intervals and correlated with the cor-responding 2-h value of subjective sleepiness, perfor-mance lapses and fastest reaction time (Pearson Product Moment Corre-lation). The resulting corre-lation coefficients were Fisher-z transformed before averaging over subjects, re-transformed and plotted against the corresponding frequency bin. Frequency specific changes in the EEG during wakefulness were correlated with changes of neurobehavioral performance. Frontal EEG power density in 0.5 Hz bins in the slow wave and theta range (1-8.5 Hz) exhibited significant positive correlations with subjective sleepiness, performance lapses and fastest reaction time (Duncan's multiple range test). In general, lower correlation coefficients were observed for the fastest reaction time vs. EEG power density. In addition, significant positive correlations were present for EEG power density in 0.5 Hz bins in the sigma-beta range (13-20 Hz). A similar 'correlation pattern' was observed for the occipital derivation. In general, correlation coefficients observed for the occipital lead were lower, and EEG power density in fewer 0.5 Hz bins showed a significant positive correlation with the neurobehavioral measures: subjective sleepiness, performance lapses and fastest reaction time.

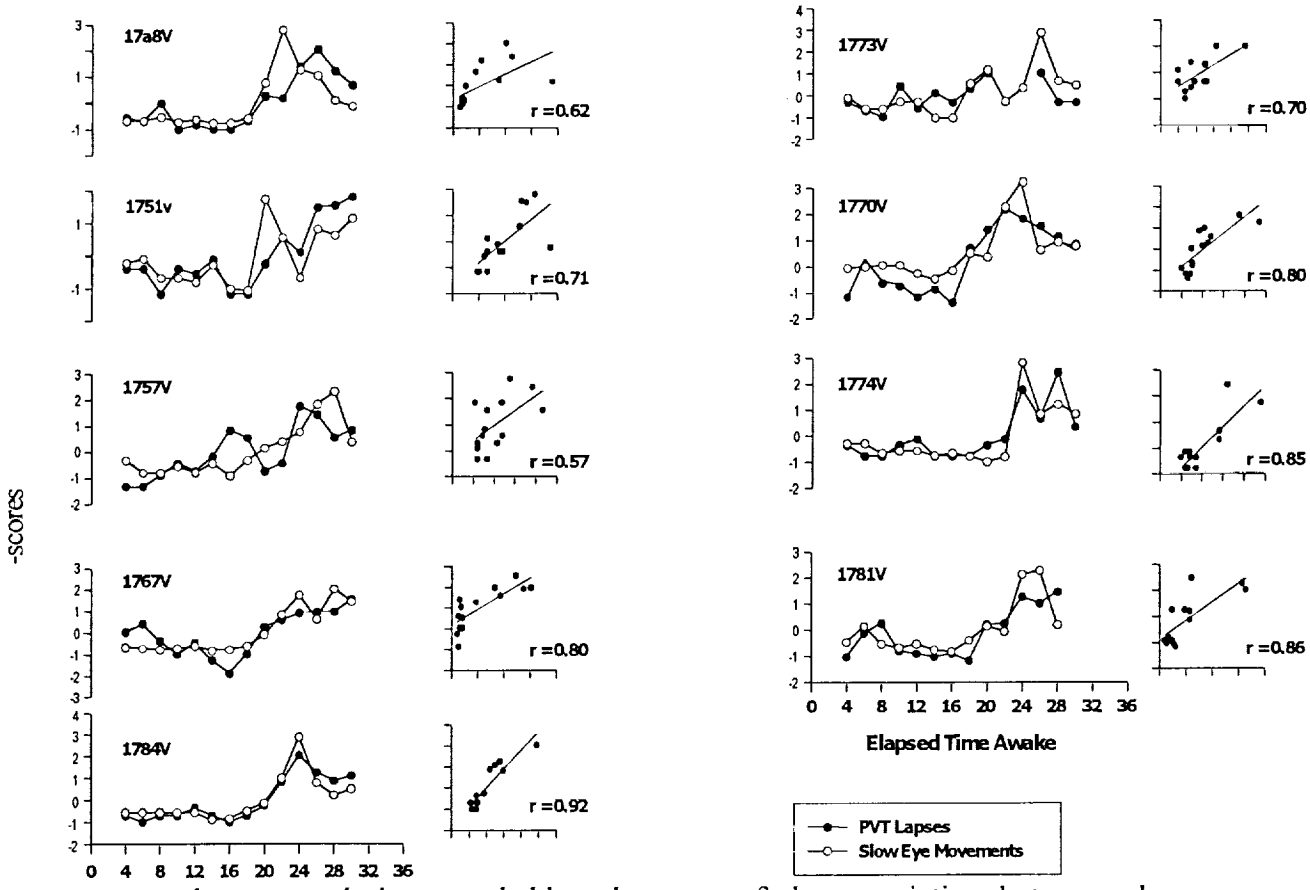


In addition, cross-correlation analyses between EEG power density in selected frequency bands and psychomotor vigilance performance have demonstrated that frontal EEG power density in the slow wave and theta band are most highly correlated with changes in performance. These data demonstrate that the correlation between EEG and neurobehavioral performance is EEG-frequency and EEG derivation specific.

c. Inter-individual differences in the time course of the incidence of slow eye movements closely resemble the inter-individual differences in the time course of neurobehavioral performance during a 28-h episode of sustained wakefulness.

We have reported a close association between ocular and EEG correlates and neurobehavioral performance. However, these associations were based on mean time courses of the incidence of slow eye movements and psychomotor vigilance performance (PVT). In order to evaluate the robustness of these associations we investigated inter-individual differences in the relationship between the incidence of slow eye movement and PVT lapses (see figure).

Slow eye movements and PVT lapses were binned in 2-h intervals and z-transformed for each subject separately during a 28-h episode of sustained wakefulness. Preliminary data of the first nine subjects indicate that inter-individual differences in the time course of the incidence of slow eye movements closely resembled the inter-individual differences in the time course of neurobehavioral performance (PVT performance lapses; > 500ms). The lowest correlation coefficient was 0.57 and the highest 0.92. Further analyses will include time-dependent aspects of this correlation . This analysis will be implemented to identify the optimal time interval to predict a performance lapse on the basis of slow-eye movements. The



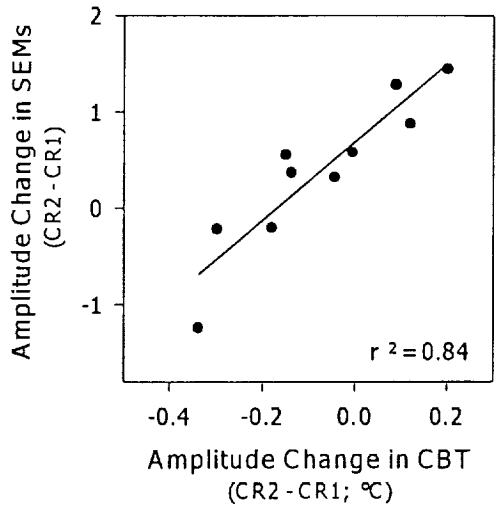
current analyses reveal the remarkable robustness of the association between slow eye movements and neurobehavioral performance.

d. Light-induced changes in the amplitude of the circadian pacemaker are associated with changes in the amplitude of the circadian modulation of alertness and the circadian modulation of the incidence of slow eye movements.

There is a strong circadian modulation of the progressive increase in the occurrence of slow eye movements (SEMs) during extended wakefulness, and changes in the occurrence of

SEMs are closely correlated with changes in subjective sleepiness. Appropriately timed exposures to bright light stimuli can induce large changes in amplitude of circadian rhythms. Reductions of the endogenous circadian amplitude after bright light exposure have been suggested to be linked to a reduced amplitude of alertness. We tested the hypothesis whether changes in the endogenous circadian amplitude elicited by different exposures to bright light stimuli are reflected in changes in the circadian modulation of SEMs, an objective measure of sleepiness.

Following three scheduled days and nights in the laboratory during which subjects slept at their habitual times, ten male subjects, age range 21-30 years, underwent a constant routine protocol (CR) which lasted up to 32h (CR1) to evaluate endogenous circadian amplitude of core body temperature (CBT) and subjective sleepiness and SEMs. The subjects were then exposed to either one, two or three bright light episodes each occurring during one circadian cycle. The bright light stimulus was followed by a final post-stimulus CR (CR2). Subjective sleepiness was assessed every 30 min on the Karolinska Sleepiness Scale (KSS) and SEMs – derived from two digitized electrooculogram recordings from the outer canthi of the right and left eye-- were visually scored per 30-s epoch and the percentage of SEMs per 5-min episodes were calculated. The 24-h window, 5 hours after scheduled waketime to CR1 and CR2, were used for the amplitude assessment of CBT, subjective sleepiness and SEMs. By applying non-linear regression analysis, the amplitude of CBT was quantified for each subject separately by fitting a sinusoidal function
consisted of a fundamental oscillation and its first harmonic. In addition to the aforementioned function, a linear component was added in order to fit the time course of subjective sleepiness and SEMs. Amplitude was defined as the composite of the fundamental component and its first harmonic for all variables.

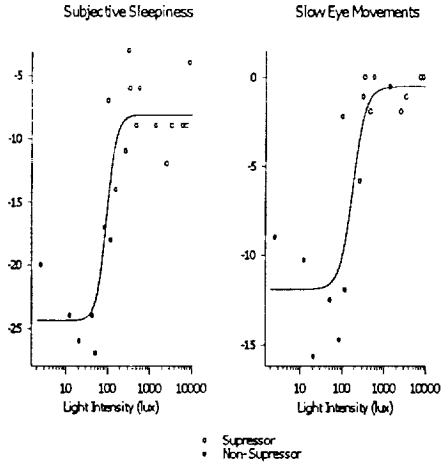


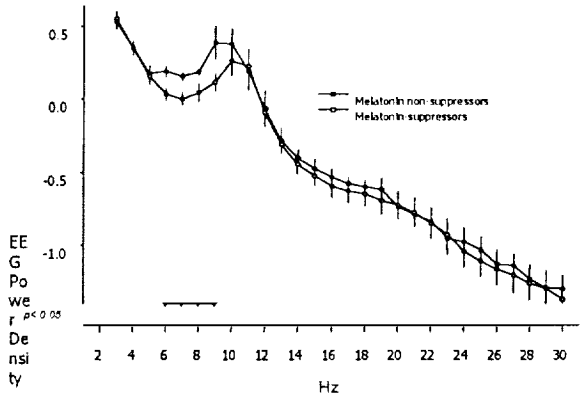
Significant correlations between the change in CBT amplitude (CR2-CR1) and the amplitude change in subjective sleepiness and SEMs (CR2-CR1) were found. SEMs (see Figure): $r^2 = 0.84$ ($r = 0.91$, $p < 0.0002$); subjective sleepiness: $r^2 = 0.85$ ($r = 0.92$, $p < 0.00016$). In addition, the correlation between the change in amplitude of SEMs and subjective sleepiness was significant: $r^2 = 0.81$ ($r = 0.9$, $p < 0.0005$). The mean percentage of SEMs per 5-min interval during CR1 was 6.6 ± 0.7 %.

The data indicate a strong relationship between the amplitude of the circadian rhythm of CBT and the amplitude of the circadian modulation of both subjective and objective measures of sleepiness, i.e. the occurrence of SEMs. This indicates that in addition to the demonstrated effects of phase on neurobehavioral function, the amplitude of the pacemaker also contributes to changes in neurobehavioral function.

e. Light-induced acute changes in alertness are associated with acute changes in the EEG and the incidence of slow eye movements during wakefulness.

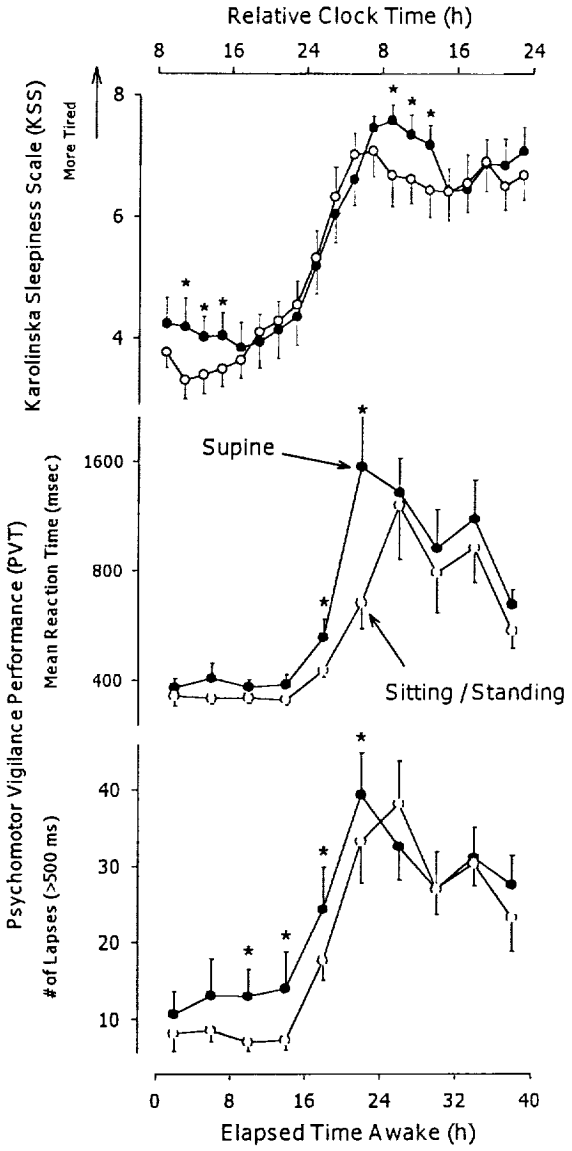
Ocular light exposure elicits both circadian and acute physiological responses in humans. In a dose response protocol in which subjects were exposed to nocturnal illuminances ranging from 3 to ~ 9000 lux for 6.5 hours during the early biological night, the acute affect of light on subjective sleepiness as well as the incidence of slow eye movements and the spectral composition of the EEG during wakefulness were assessed. Light exerted an acute subjective alerting response, reduced the incidence of slow eye movements, and reduced the EEG power density in the theta-alpha range (5-9 Hz). High positive correlations between suppression of endogenous plasma melatonin concentrations and these physiological effects were observed. The responses of all three variables to light were consistent with a logistic dose response curve. In close agreement with the sensitivity of suppression of melatonin to light, we found that half of the maximal alerting response achieved in response to bright light of ~9,000 lux can be obtained with room light of ~100 lux. The present data indicate that variations in illuminances within the range of typical, ambient, room light (90 to 180 lux) can have a significant impact on subjective alertness and its electrophysiologic concomitants in humans. These effects appear to be mediated by the light-induced suppression of plasma melatonin





f. Neurobehavioral performance decrements associated with sleep loss and circadian phase are exacerbated by a supine body position

Human cognitive performance and vigilance are modulated by an interaction of circadian and sleep-wake dependent processes. Here we investigate whether neurobehavioral performance during wakefulness is modulated by postural concomitants of wakefulness such as the upright posture, which is associated with an elevated sympathetic tone.



Following three 24-h baseline days (16h scheduled wakefulness and 8h of sleep) in which subjects slept at their habitual bedtime, 7 male and 5 female healthy adults, age range 19-28 years, underwent one of two 40-h posture modified constant routine protocols (CR), in a balanced cross-over design. Subjects were randomly assigned to start with either a sitting/standing CR or supine CR. The supine CR consisted of subjects remaining in bed with a bed angle of 0° and in a supine position, while in the sitting/standing CR subjects alternated between 40 minutes of sitting upright in a chair and 20 minutes of free standing throughout the entire 40 hours. After an 8-h sleep episode following the CR, subject were scheduled to two standard 24-h days and then underwent the other 40-h posture modified CR, i.e. either supine or sitting/standing Light levels were maintained at < 50 lux for the entire study except during sleep episodes (0 lux). Neurobehavioral performance was assessed throughout each posture modified CR with a 10-min Psychomotor Vigilance Test (PVT) every 120 min (only during the sitting portions in the sitting/standing CRs). Subjective sleepiness was assessed every 20 min on the Karolinska Sleepiness Scale (KSS). Due to a technical problem, PVT data from one subject were not included in the analysis.

In the sitting/standing condition the time course of subjective sleepiness exhibited fairly stable levels throughout the first 10 hours of wakefulness followed by an increase in sleepiness during the phase of melatonin secretion and a decline of sleepiness after approximately 26 hours of wakefulness. In the constant supine posture condition the evening increase in subjective sleepiness occurred later than in the sitting/standing condition, i.e. after about 16 hours of wakefulness rANOVA: interaction *posture x time*: $p < 0.04$). During the biological day, subjects were significantly more sleepy when in a supine position (asterisks indicate significant post-hoc comparisons, LSD test). Significant *posture x time* interactions

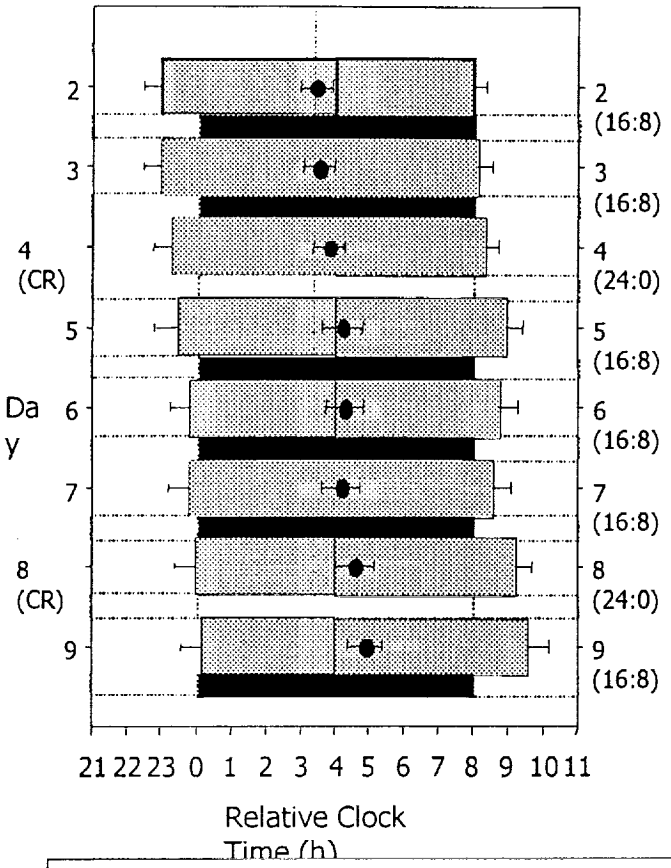
were also observed for the mean reaction times ($p<0.03$) and performance lapses (>500ms; $p<0.07$) in the PVT. In contrast to subjective sleepiness, most of the effects of body posture on PVT performance occurred during the early evening and the biological night.
The present data indicate that a constant supine posture exacerbates decrements in neurobehavioral performance in a time dependent manner, similar to the reported effects of reduced stimulation on performance during sleep deprivation[1]. Interestingly, most of the negative performance effects induced by a supine posture occurred during the biological night when subjectively rated sleepiness did not differ between the posture conditions (supine vs. sitting/standing). This dissimilar time course of subjective sleepiness and objectively evaluated psychomotor vigilance performance implies that subjective assessments are more unreliable under these conditions of supine body position.
We conclude that postural changes associated with the sleep-wake cycle reinforce the circadian and homeostatic regulation of neurobehavioral performance.

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[1]Dijkman M, Sachs N, Levine E, Mallis M, Carlin MM, Gillen KA, Powell JW, Samuel S, Mullington J, Rosekind MR, Dinges DF. Effects of reduced stimulation on neurobehavioral alertness depend on circadian phase during human sleep deprivation. Sleep Res. 27: **????** 1997.
Research supported by a grant of NASA Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute.

g. Instability of circadian phase of melatonin rhythm under low intensity ambient light conditions

The human circadian pacemaker has been shown to exhibit stability, precision and a near 24-hour period under controlled lighting conditions. Astronauts are exposed to variable rest-activity cycles and variable light-dark cycles. Such conditions may jeopardize stable entrainment. We investigated the stability of day-by-day phase information using the plasma melatonin rhythm as a marker of the human circadian pacemaker in a 12-day protocol, which included two overnight shifts under constant routine conditions in dim light (< 13 lux). In all subjects (n=10), a progressive daily drift of circadian phase to later cock time in the course of the experiment was observed. After the first overnight shift, circadian melatonin phase was delayed and did not regain the pre-constant routine phase during the following three 24-h light-dark cycles (LD; 16:8 hours; 5-13 lux during wake and 0 lux during sleep). We analyzed over the duration of the experiment the observed circadian periods ranged from 24.03 to 24.38 hours. Our data indicate that a change in the LD cycle concomitant with a change in the rest-activity cycle, may have a significant impact on the stability of the human circadian pacemaker even under light intensities more than a magnitude above the reported and predicted illuminance levels for human circadian entrainment by Kronauer's model. Whether non-photic zeitgebers such as social contacts, knowledge of clock time or food intake may act as substitute synchronizer under such an experimental setting remains to be determined. The results from this study have implications for people working in an unnatural temporal environment exposed to unusual patterns of light such as occur during spaceflight.



Day-to-day variability in the upward mean [illegible] midpoint, the downward mean [illegible] midpoint, n = 10 mean values ± 1 SEM). The

h. Neurobehavioral performance decrements associated with sleep loss and circadian phase are exacerbated by a supine body position

Human cognitive performance and vigilance are modulated by an interaction of circadian and sleep-wake dependent processes. Here we investigate whether neurobehavioral performance during wakefulness is modulated by postural concomitants of wakefulness such as the upright posture, which is associated with an elevated sympathetic tone.

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We conclude that postural changes associated with the sleep-wake cycle reinforce the circadian and homeostatic regulation of neurobehavioral performance.

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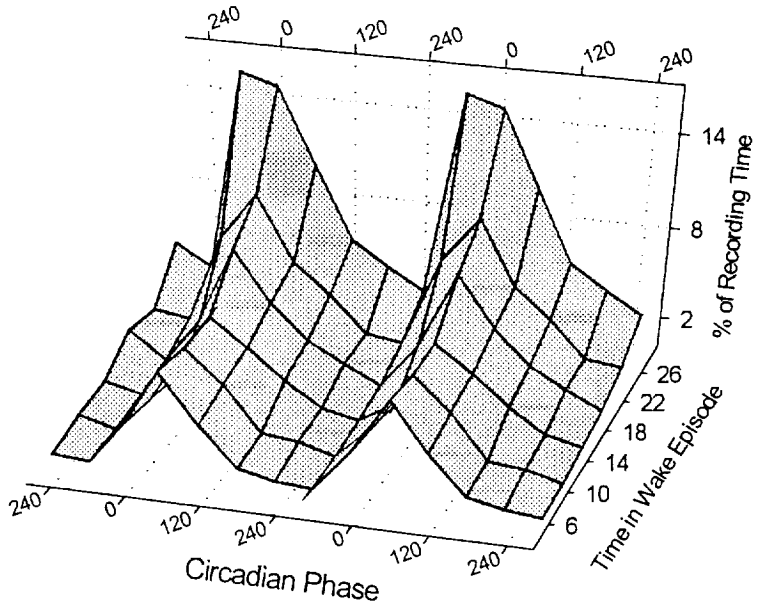
[1]Dijkman M, Sachs N, Levine E, Mallis M, Carlin MM, Gillen KA, Powell JW, Samuel S, Mullington J, Rosekind MR, Dinges DF. Effects of reduced stimulation on neurobehavioral alertness depend on circadian phase during human sleep deprivation. Sleep Res. 27: ???? 1997.

Research supported by a grant of NASA Cooperative Agreement NCC 9-58 with the National Space Biomedical Research Institute.

i. Non-linear interaction between circadian and homeostatic modulation of slow eye movements during wakefulness in humans

Sixteen men participated in a double-blind study on the effects of caffeine or placebo on performance and sleep. They were scheduled for 14 cycles of a 42.85h period (28.57h wake/ 14.28h sleep), which corresponds to 25 sidereal days. Light levels were < 0.3 lux during sleep episodes and < 20 lux for during wake episodes. EEG and two EOG signals were recorded continuously on a Vitaport-2 digital recorder. Here we report data from 7 subjects who received placebo. All 30-s epochs during hours 4 - 27 of scheduled wakefulness were visually inspected and scored by one person for the occurrence of SEMS during wakefulness and for brief episodes of sleep during scheduled wakefulness (a total of ~ 270',000 epochs). Core body temperature was collected throughout the protocol from a rectal temperature sensor, and was used to assess the circadian period and phase.

Effects of circadian phase and elapsed time awake on the incidence of SEMs during wakefulness were observed in every subject. The interaction between circadian phase and wake dependent modulation is illustrated in the Figure (mean values, n = 7). SEMs exhibited a robust circadian rhythm with highest values around or shortly after the core body temperature minimum, and lowest values around 240° which corresponds to a clock time of approximately 10 p.m. (wake-maintenance zone) during entrainment. The amplitude of the circadian modulation progressively increased with elapsed time of scheduled wakefulness. Thus SEMs were most frequent when the end of the wake episode coincided with the minimum of the core body temperature rhythm. A general increase in the incidence of SEMs throughout the 28-h wake episode was observed (homeostatic component). These observations were confirmed by statistical analyses (2-way ANOVA for repeated measures) which revealed a significant factor for circadian phase ($F_{5,30} = 15.3$, $p < 0.0001$) elapsed



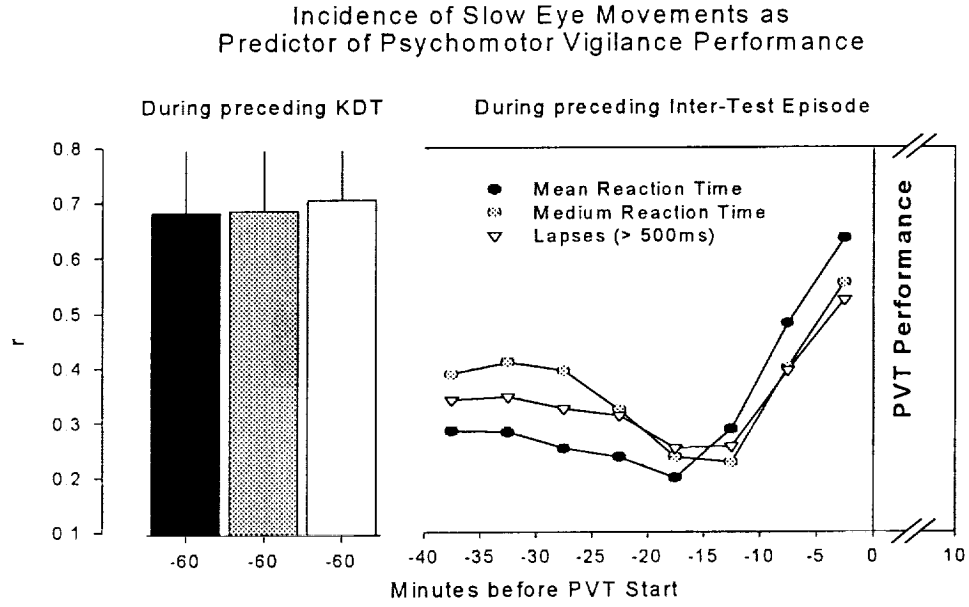
time in wake episode ($F_{5,30} = 25.9$, $p < 0.0001$) and also a significant interaction between these two factors ($F_{25,150} = 2.1$, $p = 0.025$).

These data demonstrate that variations in oculomotor control, as indexed by SEMs, exhibit circadian and homeostatic modulation, which are very similar to the observed changes in subjective sleepiness and neurobehavioral function under similar experimental conditions We also found that the circadian modulation in SEMs varies significantly with time awake, which gives further evidence for the assumption of a non-linear interaction between the circadian and homeostatic process. These data demonstrate that a critical zone of neurobiologic vulnerability occurs when extended durations of wakefulness converge with an adverse circadian phase.

j. Prediction of performance failure from EOG measures obtained prior to neurobehavioral performance testing

An essential step on the critical path to the development and implementation of countermeasures for the risks associated with neurobehavioral performance decrements is the development of methods that can predict future neurobehavioral performance decrements and performance failure. Such prediction would allow intervention prior to critical failures. We

analyzed the predictive power of the incidence of slow-eye movements prior to a psychomotor vigilance task in 9 volunteers. The volunteers (n=9) were observed during a 28-h constant routine while in a semi-recumbent posture. EOG was recorded continuously. Every hour the subjects performed a Karolinska Drowsiness test and every two hours a performance battery which included the Psychomotor Vigilance Task (PVT) (10 min duration). Correlation between lapses on the PVT (response time > 500 ms), median and mean reaction time and the incidence of slow eye movements prior to the PVT were calculated in each subject, Fisher z-transformed and then averaged. This correlation was computed for consecutive 5-min intervals preceding the PVT as well as for the 4-min portion of the KDT scheduled one hour prior to the PVT. In the last 5 minute interval prior to the PVT the correlation between the incidence of slow-eye movements, lapses and the median and mean reaction time were > 0.5. This correlation became weaker in the 5-min intervals farther removed from the performance test. However, the correlation between the incidence of slow-eye movements during the KDT scheduled one hour before the PVT and the lapses during the PVT was > 0.85. This strong association was also observed for the mean and medium reaction time (See Figure). These data imply that the future performance can be predicted from EOG parameters assessed during a 4 minute standardized KDT. Apparently, the KDT in combination with EOG measures allows objective assessment of the status of the sleep homeostat and predicts future performance.









B.

Original Reports, and Abstracts, Year 1 & 3 - funded through NSBRI funding and appropriately acknowledged

1. Cajochen C, Khalsa SBS, Wyatt JK, Czeisler CA, Dijk DJ . EEG and ocular correlates of neurobehavioral decrements associated with circadian melatonin phase and sleep loss. Am. J. Physiol. (Regulatory, Integrative, Comparative Physiol). 1999; 277:R640-R649.

2. Cajochen C, Foy R, Dijk DJ. Frontal predominance of relative increase in sleep delta and theta EEG activity after sleep loss in humans. Sleep Res. Online, 1999; 2:65-69.

3. Dijk DJ. Circadian variation of EEG power spectra in nonREM and REM sleep in humans: dissociation from body temperature. J. Sleep Res. 1999; 8:189-195.

4. Jewett ME, Wyatt JK, Ritz-De Cecco A, Khalsa SB, Dijk DJ, Czeisler CA. Time course of sleep inertia dissipation in human performance and alertness. J Sleep Res, 8 1999;1-8.

5. Jewett ME, Dijk DJ, Kronauer RE, Dinges DF. Dose response relationship between sleep duration and human psychomotor vigilance and subjective alertness. Sleep, 22 1999;171-179.

6. Wyatt JK, Ritz-DeCecco A, Czeisler CA, Dijk D-J. Circadian temperature and melatonin rhythms, sleep and neurobehavioral function in humans living on a 20-h day. Am. J. Physiol. (Regulatory, Integrative, Comparative Physiol). 1999; 277:R1120-1135.

7. Zeitzer JM, Dijk D-J, Kronauer RE, Brown RE, Czeisler CA. Sensitivity of the human circadian pacemaker to nocturnal light: melatonin phase resetting and suppression. J. Physiol.-London, 2000; 526.3 695-702.

8. Cajochen C, Zeitzer JM, Czeisler CA, Dijk D-J. Dose-response relationship for light exposure and ocular and electroencephalographic correlates of human alertness. Beh. Brain Res. 2000; 115:75-83.

Reviews/Commentaries

1. Dijk D-J, Kronauer RE. Models of sleep-regulation: successes and continuing challenges. J. Biol. Rhythms 1999; 14:569-573.

2. Dijk D-J, Jewett ME, Czeisler CA, Kronauer RE. Non-linear interactions between circadian and homeostatic processes: models or metrics? J. Biol. Rhythms. 1999; 14:604-605.

Abstracts

1. Cajochen C, Khalsa SBS, Czeisler CA, Dijk D-J. Circadian Variation of Slow Eye Movements during sustained Wakefulness in Humans. Abstract Presented at the Society for Research on Biological Rhythms, Amelia Island FL., May 1998.

2. Cajochen C, Khalsa SBS, Czeisler CA, Dijk D-J Time course of EEG power density

during wakefulness and subjective sleepiness during a 24-h constant routine. Abstract for American Professional Sleep Societies meeting, New Orleans, June 1998. Sleep 21:243,1998.

3. Cajochen C, Wyatt JK, Khalsa SBS, Czeisler CA, Dijk D-J. Circadian and homeostatic variation of EEG activity during extended wakefulness. European Sleep Research Society Meeting, Madrid, Spain, September 1998, J. Sleep Res. 1998 7 (S2): p 35.

4. Cajochen C, Khalsa SBS, Ehrlich DA, Czeisler CA, Dijk D-J. Changes in circadian modulation of slow eye movements are correlated with changes in endogenous circadian amplitude. APSS99, Orlando FL, Sleep 22 1999 (S): p 143.

5. Cajochen C. Zeitzer JM , Czeisler CA, Dijk D-J. Dose-response relationship for light intensity and alertness and its ocular and EEG correlates. Third International Congress of the World Federation of Sleep Research Societies, Dresden, Germany, October 5-9, 1999. Sleep Res. Online, 1999 2 (S1):517.

6. Cajochen C, Martens WM, Dijk D-J. Topographical aspects of the effect of sleep deprivation: a comparison of time averaged spectral analysis (FFT) and instantaneous frequency analysis of the EEG. Third International Congress of the World Federation of Sleep Research Societies, Dresden, Germany, October 5-9, 1999. Sleep Research Online, 1999 2 (S1):16.

7. Czeisler CA, Dijk D-J, Neri DF, Hughes RJ, Ronda JM, Wyatt JK, West JB, Prisk GK, Elliott AR, Young LR. Ambient light intensity, actigraphy, sleep and respiration, circadian temperature and melatonin rhythms and daytime performance of crew members during space flight on STS90 and STS-95 missions. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999- League City, Texas, pp 347-349 Supplement Abstracts pp 51-52.

8. Dijk D-J, Cajochen C. Electroencephalographic and ocular correlates of neurobehavioral performance decrements. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999- League City, Texas, pp 347-349.

9. Dijk D-J. Human Sleep-wake regulation, aging and the electroencephalogram: a circadian perspective. Abstract for Workshop 'The regulation of sleep' Human Frontier Science Program, Strassbourg May 31-June2, 1999.

10. Foy R, Cajochen C, Dijk D-J. Frontal predominance of increase in sleep delta activity after sleep loss in humans. American Professional Sleep Societies , Orlando FL, Sleep 1999; 22 (S): p 147.

11. Khalsa SBS, Dijk D-J. Melatonin as a countermeasure for entrainment to the sleep/wake schedules required during shuttle missions. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999- League City, Texas, pp 347-349 Supplement Abstracts pp 56-59.

12. Dijk D-J. Circadian and homeostatic components of sleep consolidation, REM sleep and

the electroencephalogram. American Physiological Society Conference on Determinants of Vigilance: Interaction between the Sleep and Circadian Systems, Ft. Lauderdale, FL, October 19-22, 1999.

13. Dijk D-J. Sleep, Circadian rhythms and space flight: flight and ground based studies. Abstract for Conference: Futures in UK Space Biomedical Research. University College of London, British National Space Centre. December; 1999.

14. Wyatt JK, Dijk D-J, Ritz-de Cecco A, Ronda JM, Czeisler CA. Low-dose, repeated caffeine administration as a countermeasure to neurobehavioral deficits during a forced desynchrony protocol. Abstract APSS 14th Annual Meeting, Las Vegas Nevada June 17-22, 2000. Sleep 23 Supplement 2. A45.

15. Ehrlich DA, Cajochen CA, Dijk D-J. Neurobehavioral performance decrements associated with sleep loss and circadian phase are exacerbated by a supine body position. Abstract for 14th Annual Meeting of American Professional Sleep Societies, Las Vegas Nevada June 17-22 2000. Sleep 23 S2. A72-A73.

16. Cajochen C, Wyatt JK, Bonikowska M, Czeisler CA, Dijk D-J. Non-linear interaction between circadian and homeostatic modulation of slow eye movements during wakefulness in humans. Abstract,15th Congress of the European Sleep Research Society, Istanbul, Turkey, September, 12-16, 2000. J Sleep Res 2000; 9; S1:p29

Presentations

D-J Dijk. Melatonin as a Hypnotic for Neurolab Crew. Invited presentation. Symposium: Neurolab, A Space Shuttle Mission Devoted to Neuroscience Research. October 1997 New Orleans, LA

D-J Dijk. Electroencephalographic and Ocular Correlates of Neurobehavioral Performance Decrements. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999, League City, Texas .

D-J Dijk. Melatonin as a hypnotic for Neurolab crew. Neurolab Scientific Results Symposium, April 14-16 1999, Washington, DC .

D-J Dijk. Human sleep-wake regulation, aging and the electroencephalogram: a circadian perspective. Invited presentation for the workshop 'The regulation of sleep' Human Frontier Science Program, May 31- June 2, 1999 Strassbourg, France.

D-J Dijk. Sleep in space: a circadian perspective. Invited presentation 20th Annual International Gravitational Physiology Meeting 6 - 11 June, 1999 Orlando, FA

D-J Dijk. Sleep and circadian rhythms in astronauts on Neurolab. Invited presentation for the symposium 'Astronauts/cosmonauts: sleep and rhythms'. 13th annual meeting of the APSS June 19-24 1999, Orlando, FA.

D-J Dijk Circadian and homeostatic components of sleep consolidation, REM sleep and the

electroencephalogram. Invited presentation APS conference on Determinants of Vigilance: Interaction Between the Sleep and Circadian Systems. October 19-12, 1999; Ft Lauderdale, FA

Dijk, D-J. Sleep, Circadian Rhythms and Spaceflight: Flight and Ground Based Studies. Futures in UK Space Biomedical Research conference. University College of London, British National Space Centre. December 3rd 1999.

Dijk D-J. Quantitative Monitoring of Vigilance: EEG and EOG Decrements of Neurobehavioral Performance Decrements. Retreat of the National Space Biomedical Research Institute, January 10-13, 2000 Montgomery, Texas

Dijk, D-J. Circadian Regulation of Sleep: Laboratory and Space Studies. Defence Evaluation and Research Agency, Human Sciences, March 3rd, 2000 Farnborough, UK.

Dijk, D-J. Circadian Regulation of Human Sleep: A Role for Melatonin? Gordon Research Conference: Pineal Cell Biology, Oxford, UK 27-31 August, 2000.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report
October 1, 1997 to September 30, 2000

1. **Research Team: Human Performance Factors, Sleep and Chronobiology Team**

2. **Project Name: Project 4: On-Line Analysis of Physiologic and Neurobehavioral Variables During Long-Duration Space Missions**

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Date:

EXECUTIVE SUMMARY

BACKGROUND

The goal of this project is to develop reliable statistical algorithms for on-line analysis of physiologic and neurobehavioral variables monitored during long-duration space missions. Maintenance of physiologic and neurobehavioral homeostasis during long-duration space missions is crucial for ensuring optimal crew performance. If countermeasures are not applied, alterations in homeostasis will occur in nearly all-physiologic systems. During such missions data from most of these systems will be either continually and/or continuously monitored. Therefore, if these data can be analyzed as they are acquired and the status of these systems can be continually assessed, then once alterations are detected, appropriate countermeasures can be applied to correct them.

One of the most important physiologic systems in which to maintain homeostasis during long-duration missions is the circadian system. To detect and treat alterations in circadian physiology during long duration space missions requires development of: 1) a ground-based protocol to assess the status of the circadian system under the light-dark environment in which crews in space will typically work; and 2) appropriate statistical methods to make this assessment. The protocol in **Project 1, Circadian Entrainment, Sleep-Wake Regulation and Neurobehavioral** will study human volunteers under the simulated light-dark environment of long-duration space missions. Therefore, we propose to develop statistical models to characterize in near real time circadian and neurobehavioral physiology under these conditions.

The specific aims of this project are to test the hypotheses that: 1) Dynamic statistical methods based on the Kronauer model of the human circadian system can be developed to estimate circadian phase, period, amplitude from core-temperature data collected under simulated light-dark conditions of long-duration space missions. 2) Analytic formulae and numerical algorithms can be developed to compute the error in the estimates of circadian phase, period and amplitude determined from the data in Specific Aim 1. 3) Statistical models can detect reliably in near real-time (daily) significant alternations in the circadian physiology of individual subjects by analyzing the circadian and neurobehavioral data collected in **Project 1**. 4) Criteria can be developed using the Kronauer model and the recently developed Jewett model of cognitive performance and subjective alertness to define altered circadian and neurobehavioral physiology and to set conditions for immediate administration of countermeasures.

RESEARCH PLAN YEARS 2AND 3

At the outset of Year 2 we made three changes in the research plan as a consequence of the research findings in Year 1 and the recommendations of the review committee.

Change 1: Dynamic Assessments of Circadian Phase from Forced Desynchrony Studies. In our Year 1 research plan, our original goal was to use the data collected during the 25 24 hour days of core-temperature data collected from **Project 1** to develop a technique for making dynamic assessments of circadian phase. These estimates would provide the circadian input to the performance and subjective alertness model prediction developed by Dr. Jewett. Our original hypothesis was that under low light conditions these subjects would free run and therefore these data would provide an excellent framework for making dynamic assessments of circadian phase.

All of the 3 subjects analyzed by the end of Year 1 were entrained during the 25 24 hour day. Our analysis and the independent constant routine assessments confirmed this. Therefore to test the ability of our analytic framework to make dynamic assessments of circadian phase we use the temperature data from the forced desynchrony part of the protocol. During this phase of the protocol the subject is desynchronized from the 28-hour day.

Change 2: Average Prediction of Performance and Subjective Alertness. In our Year 1 research plan our original goal was to develop straight away an algorithm for making time specific individual predictions of performance and subjective alertness using the models developed by Dr. Jewett. We realized that moving directly to individual predictions was too large an initial step. Therefore we will use the performance, alertness and circadian phase data to first adapt the Jewett model to predict average performance, since this is what it was initially developed to predict. Once the model shows good predictions with average performance and subjective alertness, we will then return the problem of individual predictions.

Change 3: Using the Expertise of a Neurobehavioralist on the Project 4. Our scientific review committee recommended that we include a neurobehavioralist on our team in order to better focus the work on performance and subjective alertness. In response to this suggestion, we have Dr. Megan Jewett working on this component of the modeling for the project. She developed the performance and subjective alertness models for her Ph.D. dissertation and has been working with us to adapt them to the study of the subjects on the simulated long-duration space missions.

The objective in Year 3 was to analyze the core-temperature, performance and alertness data of the 7 subjects in the control group from Project 1.

PROGRESS, RESULTS AND IMPLICATIONS FOR FUTURE RESEARCH

Core-Temperature Analysis. The methods were applied to the 7 control subjects studied in **Project 1**. We have successfully used our methods to analyze core-temperature data on the forced desynchrony protocol and demonstrate that the period of the human circadian pacemaker is closer to 24 instead of 25 hours. We published these findings in *Science* in July of 1999. In addition we published two manuscripts detailing our methods for dynamic assessment of circadian phase.

Genetic Algorithm. We made the genetic algorithm a standard part of our analysis framework. It has been implemented with a continuous state discrete-time Kalman filter algorithm in order to fit unevenly spaced core-temperature data.

New Model for Core-Temperature. The new core-temperature model described in our two publications holds promise for giving a better description of the dynamics of the human circadian pacemaker. This description can be further enhanced if realistic models of the thermoregulatory and activity interactions with the circadian pacemaker can be characterized. We will work on developing these model components.

Analysis of Performance and Subjective Alertness. We are completing our analysis of subjective alertness and performance for all of the subjects in the control group in **Project 1.** A manuscript on this work in under preparation.

Growth Hormone Model. We have developed a growth hormone model so that we can now include the negative feedback to measure the effect of growth hormone plasma levels on its own secretion. We are using the model to analyze normal growth hormone physiology in normal subjects.

Cortisol Model. We have also submitted for publication a manuscript detailing a new stochastic differential equation model of plasma cortisol levels. This model may be used to analyze diurnal cortisol patterns as well as serving as a starting point for extending our work on growth hormone to the analysis of melatonin series with more than one secretory event.

Implications for Future Research. We have developed more accurate statistical models of human circadian marker rhythms including core-temperature, growth hormone and cortisol. These new models provide an accurate means of assessing characterizing circadian physiology with respect to standard marker rhythms in both space and non-space related research. We have used the dynamic phase information from the core-temperature model as an input to the Jewett performance and subjective alertness models. The paradigm may be useful for developing accurate strategies to monitor the circadian health, neurobehavioral state of astronauts during long-duration space missions and for implementing and measuring the effects of countermeasures when significant alterations in these states are detected.

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3. PROJECT RESEARCH ACTIVITY

A. Hypotheses, Objectives and Specific Aims from Original Proposal

Specific Aims

The specific aims are to test the hypotheses that: 1) Physiologically-based statistical models can be developed to estimate circadian phase, period, amplitude from core-temperature, melatonin, neurobehavioral and actographic data collected under simulated conditions of long-duration space missions. 2) Analytic formulae and numerical algorithms can be developed to compute the error in the estimates of circadian phase, period, amplitude determined from the data in Specific Aim 1. 3) Statistical models can detect reliably in near real-time significant changes in the circadian physiology of individual subjects by analyzing the circadian and neurobehavioral data collected in Projects 1 and 3. 4) Criteria can be developed from the analyses in Specific Aim 3 to define abnormal circadian and neurobehavioral physiology and to set conditions for immediate administration of countermeasures. Moreover, the circadian model estimates may be input to behavioral models developed by us to quantify both the extent of behavioral abnormalities and the effectiveness of their countermeasures.

Background and Rationale

Maintenance of physiologic and neurobehavioral homeostasis during long-duration space missions is crucial for ensuring optimal crew performance. If countermeasures are not applied, alterations in homeostasis will occur in nearly all-physiologic systems. During such missions data from most of these systems will be either continually and/or continuously monitored. Therefore, if these data can be analyzed as they are acquired and the status of these systems can be continually assessed, then once derangements are detected, appropriate countermeasures can be applied to correct them.

One of the most important physiologic systems in which to maintain homeostasis during long-duration missions is the circadian system. The alterations in the circadian system will occur due to loss of contact with the normal geophysical light-dark cycles. Assessing the status of the circadian system is an especially challenging task since this normally requires special protocols such as the constant routine, free-run and forced desynchrony. Under these conditions the status of the circadian oscillator is assessed by statistical analyses of phase, amplitude and period of circadian marker rhythms such as core-temperature, cortisol, melatonin, mental alertness and actographic data. The use of these protocols on long-duration space missions is not possible since they would disrupt crew work schedules. Therefore, the status of the circadian system must be evaluated from circadian variables measured under normal crew working conditions and appropriate statistical methods must developed to make this assessment.

Current mathematical models used to study the human circadian pacemaker have been developed primarily for the analysis of human core-temperature data under the three protocols mentioned above. These models divide into two categories: those designed to study the dynamical properties of the pacemaker using differential equation based simulations and those designed for static analyses of experimental data using harmonic regression models. The dynamical behaviors characterized by the simulation studies are the response of the circadian pacemaker to light, its rate of decay to its limit cycle and its response to the rest-activity cycle

(Klerman, et al, 1996). The static properties characterized by the harmonic regression analyses are the phases, period and amplitude of the pacemaker observed under stable oscillatory conditions (Brown and Czeisler, 1992). Formal statistical methods have not been used in simulation studies and therefore, the uncertainty in the inferences based on the differential equation models and their sensitivity to model specification and parameter estimation error cannot be evaluated. The harmonic regression models allow formal statistical analysis of static but not the dynamical features of circadian oscillator.

From these analysis we realize that the essential features of the core-temperature rhythm to characterize are the circadian oscillation, the thermoregulatory response, the direct effect of light and activity on the circadian oscillator and the evoked effects of activity on the observed rhythm. For the circadian variables studied **Project 1** we propose to develop statistical models to characterize their essential physiologic properties including their responses to light and activity during ground-based simulations of long-duration space missions. We propose to design the models so they can assess both static and dynamic properties of the circadian system.

B. Modification Required & Rationale for Modifications

In our Year 1 research plan our original goal was to use the core-temperature data collected during the 25 24 hour day protocol in **Project 1** to develop a technique for making dynamic assessments of circadian phase. These estimates would provide the circadian input to the performance and subjective alertness model prediction developed by Dr. Jewett. Our original hypothesis was that under low light conditions these subjects would free-run and therefore these data would provide an excellent framework for making dynamic assessments of circadian phase. All of the three subjects analyzed by Year 1 were entrained during the 25 24 hour days. Our analysis and the independent constant routine assessments confirmed this. Therefore to test the ability of our analytic framework to make dynamic assessments of circadian phase, we use the temperature data from the forced desynchrony part of the protocol. During this phase of the protocol, the subject's circadian system is desynchronized from the 28-hour day.

Another goal in our Year 1 research plan was to develop an algorithm for making time-specific individual predictions of performance and subjective alertness using the models developed by Dr. Jewett. We realized that moving directly to individual predictions was too large an initial step. Therefore, in Year 2 we use the performance, alertness, and circadian phase data to first adapt the Jewett model to predict average performance and alertness since this is what it was initially developed to predict. Once the model shows good predictions for average performance and subjective alertness, we will return to the problem of individual predictions.

C. Summary of Progress in Year 1

The first significant accomplishment of Year 1 was establishing a seamless data transfer system between the Statistics Research Laboratory at the Massachusetts General Hospital and the Circadian and Sleep Disorders Medicine Laboratory at the Brigham and Women's Hospital. The Kronauer model given by Eq. 2 below as well as harmonic regression analysis were used to dynamically assess circadian phase and amplitude in three subjects studied in **Project 1**. For the 24-hour protocol, we developed a procedure for estimating at each day possible drifts in the phase and amplitude of the circadian oscillator. We initiated our study of Walsh function based

representation of the activity induced evoked effects. We also conducted extensive studies of the correlations in the thermoregulatory response and determined that AR(1) and AR(2) models provided highly efficient summaries of the thermoregulatory component of the model.

D. Action Taken in Response to Year 1 Critique

Our scientific review committee recommended that we include a neurobehavioralist on our team in order to better focus the work on performance and alertness. In response to this suggestion, we have Dr. Megan Jewett collaborating with us on the project. She developed the performance and alertness models as part of her Ph.D. dissertation, and she has been adapting them to study the subjects on the simulated light conditions of long-duration space missions from **Project 1**.

4. DETAILED RESEARCH PLAN – YEARS 2 AND 3

Specific Aim 1

For the simulated light-dark environment of the long-duration space missions we have postulated the following model for core-temperature data. We assume that core-temperature data $y_{t_1},\ldots,y_{t_N}$ are collected in the interval $[0,T]$ where $t_n = n\Delta t$, $n = 1,2,\ldots,N$ and $N\Delta t = T$. The core-temperature measurement may be expressed as

$$y_{t_n} = \mu + s_{t_n} + x_{t_n} + v_{t_n} \tag{1}$$

where μ is the core-temperature mean, s_{t_n} is the circadian oscillation, x_{t_n} is the evoked effect of the subject's activity level and v_{t_n} is the fluctuation in core-temperature measurements due to the body's thermoregulatory response. The variable s_{t_n} is represented as the solution to the modified van der Pol equation defined in Kronauer (1990),and Klerman et al. (1996) as

$$\begin{aligned} \frac{ds(t)}{dt} &= z(t) + \varepsilon\left(\frac{2\pi}{\tau}\right)\left[s(t) - \frac{4s(t)^3}{3\gamma^2}\right] + \left(\frac{2\pi}{\tau}\right)(1 - ms(t))CI(t)^{1/3} \\ \frac{dz(t)}{dt} &= -\left(\frac{2\pi}{\tau}\right)^2 s(t) + z(t)\frac{(1 - ms(t))CI(t)^{1/3}}{3} \end{aligned} \tag{2}$$

where τ is the intrinsic period of the oscillator, γ is the limit cycle amplitude, ε is the internal stiffness parameter, $I(t)$ is the physical intensity of the ambient light at time t, m is the circadian light modulation index, and C is a constant of proportionality. Eq. 2 makes explicit the direct effect of ambient light on the circadian oscillator and we term it the Kronauer model. If s_{t_n} is represented as a harmonic regression model then it takes the form

$$s_{t_n} = \sum_{r=1}^{d} A_r \cos\left(\frac{2\pi r t_n}{\tau}\right) + B_r \sin\left(\frac{2\pi r t_n}{\tau}\right) \tag{3}$$

where d, the number of harmonics is either 2 or 3. The choice of $d=2$ follows from the harmonic regression analysis of core-temperature data under the constant routine protocol (Brown and Czeisler, 1992). The choice of $d=3$ makes the harmonic regression model equivalent to the asymptotic series expansion in powers of ε of the van der Pol oscillator (Brown, Choe, Luithardt and Czeisler, 2000).

The form of x_{t_n} depends on the circadian protocol. For the forced desynchrony protocol with a 28-hour light-dark cycle the regular, square wave shape of x_{t_n} is well described by the harmonic regression model

$$x_{t_n} = \sum_{r=1}^{4} C_r \cos\left(\frac{2\pi r t_n}{28}\right) + D_r \sin\left(\frac{2\pi r t_n}{28}\right) \tag{4}$$

(Brown, Solo, Choe and Zhang , 1997). For the self-selected timing of activity during the free-run protocol x_{t_n} is well represented as

$$x_{t_n} = \begin{cases} \beta X(t_n) & where\ X(t_n)=1,\ if\ I(t_n)>0 \\ 0 & otherwise \end{cases} \tag{5}$$

Under the constant routine protocol the subject's activity is kept to a minimum so that $x_{t_n}=0$ for all t_n. The random variable v_{t_n} is a discrete sample from a continuous AR(1) process and is defined as

$$v_{t_n} = e^{-\alpha \Delta t} v_{t_{n-1}} + \eta_{t_n} \tag{6}$$

where α^{-1} is the time-constant for the thermoregulatory response and the η_{t_n} are independent, Gaussian random variables with zero mean and variance σ_η^2.

To address specific aim 1 we studied systematically specific modifications of each of these 3 components to determine which best describe the core-temperature data being collected in **Project 1**.

Circadian Signal. We used harmonic regression methods and the Kronauer model to represent the circadian signal component of the model.

Evoked Effect of Activity. We have employed harmonic regression methods as well as a Walsh function based representation to describe the evoked effects of activity on core-temperature. The standard definition of the Walsh functions is :

$$
\begin{aligned}
&w_j(x) = 0 \text{ for } x < 0 \text{ and } x > 1 \\
&w_0(x) = 1 \text{ for } 0 \le x \le 1 \\
&w_{2j}(x) = w_j(2x) + (-1)^j w_j[2(x - \frac{1}{2})] \\
&w_{2j+1}(x) = w_j(2x) - (-1)^j w_j[2(x - \frac{1}{2})](j = 0,1,2,\ldots)
\end{aligned}
\tag{7}
$$

These functions are extended over all real numbers by mapping the interval [0,T], where T is the period of the function being approximated into the interval [0,1] and then defining the Walsh function arguments in modulo 1.

The Walsh functions are step functions, which form an orthonormal basis analogous to a Fourier basis. We hypothesize that a low order Walsh decomposition is a more realistic approximation to the evoked effect of activity because their shape more closely resembles the shape of the activity evoked component. By performing separate decompositions of the evoked effects during wake and sleep periods with separate sets of Walsh functions phase information about the evoked effect is captured that can facilitate model fitting. This approach potentially eliminates the spectral overlap between the circadian oscillator and the fundamental of the Walsh expansion. These ideas will be extensively investigated.

Thermoregulatory Response. To date we have implemented mainly AR(1) models of the thermoregulatory component, but we will study whether higher order autoregressive moving average (ARMA) models might better characterize thermoregulation. We have found that the AR(2) model consistently gave the best fit to the core-temperature data. We plan to implement an algorithm to carry out regular AR(2) model fitting.

Specific Aim 2

We are developing for each modification of the model the analytic and numerical algorithms to compute standard error estimates for the circadian phase, amplitude and period. To carry out these calculations we are using the analytic methods developed in Brown (1990), Brown, Solo, Choe, Zhang (1997) and Brown, Choe, Luithardt, and Czeisler (2000). We will study Monte Carlo based methods for parameter fitting based on genetic algorithms.

Specific Aim 3

We studied that hypothesis that we can characterize dynamically changes in circadian phase Harmonic regression and Kronauer models will be used to analyze the core-temperature series.

Specific Aim 4

We used the estimate of circadian phase obtained from the current form of the Kronauer model as input to the Jewett performance model to make predictions about performance. The Jewett performance model represents the performance P as a function of time as

$P(t) = C(s,z,H) + H(s,z,t) + W(C,H)$ where s and z are the state variables in the Kronauer model whereas H, C, and W are the homeostatic, circadian, and sleep inertia functions respectively. Once the Kronauer model state variables are supplied, the C, H, and W functions are computed as the solutions of a coupled set of ordinary differential equations.

5. PROGRESS, RESULTS AND IMPLICATIONS FOR FUTURE RESEARCH

A. CORE-TEMPERATURE ANALYSIS

Our temperature models were fit to both the 25 24 hour day and forced desynchrony protocols described in **Project 1**. The entrainment or partial entrainment of the subjects during the 25 24-hour day protocol has up to now required static, harmonic regression methods to analyze these data. The differential equation based model have been fit to the forced desynchrony protocol data. *The core-temperature data of six of the seven subjects studied to date in **Project 1** have been cleaned of artifacts and analyzed with our methods.* We have published two major papers detailing the use of theory of these methods (Brown and Luithardt, 1999; Brown et al., 2000). We have also used the methods to correctly estimate the intrinsic period of the human biological clock (Czeisler et al., 1999).

Walsh Function Analysis of the Evoked Effects of Activity. The proximity of the circadian and evoked effect frequencies makes it difficult to separate these components with harmonic regression methods. We have tested our hypothesis that a Walsh function decomposition of the evoked component (separately taken over the wake and sleep intervals) would achieve a better separation. This hypothesis is motivated by the square wave shape of the evoked effects of the activity and that of the Walsh functions. This approach is further supported by the fact that the Walsh decomposition actually supplies phase information about the evoked effect, since its construction is asymmetric about the midpoint of the 24-hour interval. *Preliminary results, while promising, show that the Walsh functions do not completely separate the circadian and activity components when the subjects are nearly identical as under entrained conditions. Under non-entrained conditions, the Walsh decomposition should prove quite powerful.*

Harmonic Regression Analysis of Circadian Phase. Despite our inability to separate completely circadian phase and evoked effects, our initial scheme for detecting the possible drift of the circadian phase using the first harmonic (containing most of the circadian information) of a regression in one fundamental frequency has proven to be valid. This is because a drift of this first harmonic most likely follows from nonentrainment, since the evoked effects remain approximately constant due to the rigorously enforced experimental protocol. We have conducted a daily phase analyses of the subjects on the 25 24-hour day protocol. We discovered that five of the subjects maintained entrainment during the 25 days, but one of the subjects clearly began to free-run. The free-run was evident from simple examination of a full 25-day time series plot. A graphical analysis scheme would only detect a free-run state after several days. Our daily amplitude and phase analysis detected the free-run on day 6. The amplitude decreased steadily throughout the entire study. *We conclude that the feasibility of the daily harmonic analysis as an early warning scheme for detecting circadian phase changes is plausible.*

Forced Desynchrony Analysis. During forced desynchrony, the periods of the circadian oscillator (nearly 24 hours) and the activity evoked effects (28 hours) are sufficiently separated so that reliable parameter estimates of the Kronauer model, the forced desynchrony and thermoregulatory components were obtained for 5 of the six subjects. Model fitting for the 6th subject proved difficult because of numerical instability problems. Two of the subjects were studied at light levels of 15 lux whereas the other 3 were studied at less than 5 lux. *From this analysis we are able to make reliable dynamic assessments of circadian phase at approximately 10 days after the start of the forced desynchrony condition.* The state variables of the Kronauer model provided input to the Jewett performance and alertness models. (See below **in Section III C.**)

In a series of healthy young and elderly subjects we have successfully used our methods to analyze core-temperature data on the forced desynchrony protocol and demonstrate that the period of the human circadian pacemaker is closer to 24 rather than 25 hours. These findings appeared in a manuscript published in (Czeisler et al, 1999; Brown et al., 2000).

B. Genetic Algorithm and Light Model Respecification

Proper understanding of the interaction of light with the phase resetting mechanism of the circadian oscillator requires a dynamical description such as given by the Kronauer model. We have successfully implemented a maximum likelihood (ML) parameter procedure for this model and used it to analyzed core-temperature data collected on forced desynchrony protocols. We realize that an important improvement in our modeling effort can be achieved by improving our ML algorithm and/or by refining the structure of the Kronauer model to eliminate parameter indeterminacies. We have pursued both of these options, and these efforts have yielded promising results with regard to improving the Kronauer model to temperature data. We discuss these new efforts after first summarizing the current difficulties in using the Kronauer model as data analysis tool.

Reformulation of the Light Input to the Kronauer Model. The dynamical model for the circadian temperature component was given by Eq. 2. This model contains one parameter serving as a proportionality constant c between the light intensity $I(t)$ and the light driving terms for the two dynamical variables $s(t)$ and $z(t)$. While this light driving was adequate to achieve an initial fitting of model parameters to data, the work of Kronauer and Jewett (1998) suggests that an accurate description of the light effects might be achieved through introduction of separate light proportionality constants c_1 and c_2 for each dynamical variable. The new dynamical system becomes:

$$\frac{ds}{dt} = z + \varepsilon\left(\frac{2\pi}{\tau}\right)\left(s - \frac{4}{3\gamma^2}s^3\right) + \left(\frac{2\pi}{\tau}\right)(1-ms)c_1 I(t)^{\frac{1}{3}}$$

$$\frac{dz}{dt} = -\left(\frac{2\pi}{\tau}\right)^2 s + \left(\frac{2\pi}{\tau}\right)z(1-ms)c_2 I(t)^{\frac{1}{3}} \qquad (8)$$

A Genetic Algorithm: An Alternative to Newton's Method for Maximum Likelihood Estimation. Fitting this model, and the mathematical representations of the evoked temperature and correlated noise effects, to data requires the estimation of at least 18 parameters: eight parameters from the differential equations; nine parameters from a four harmonic expansion of the evoked effect; and one parameter from the AR(1) representation of the noise. Refinement of the modeling of the evoked temperature contribution (e.g. by use of piece-wise, Walsh decomposition) and/or the correlated noise (e.g. by use of higher order autoregression) would further increase the complexity of a numerical algorithm for model parameter estimation.

Our current approach to the ML parameter estimation problem involved a decomposition of the parameters into ones entering the model linearly (from the evoked contribution) and those entering nonlinearly (from the Kronauer model and AR component) into the likelihood function. The overall optimization was achieved by maximizing the likelihood with respect to the nonlinear components then solving for the linear parameters as a function of the nonlinear ones. The nonlinear parameters are updated via a quasi-Newton method, and the linear parameters are updated by solving the resulting linear system via generalized least squares using the Kalman filter. The quasi-Newton method ranks among the most sophisticated standard optimization methods. However, it has the shortcoming that it may fail to find a global maximum if the likelihood has local minima or has regions with vanishing curvature, i.e., regions that are flat. Moreover, for the specific system we are considering, further numerical instabilities may be introduced with solution of the linear system. Both of these problems become more pronounced as one increases the number of parameters in the likelihood function. The unexpectedly low values of the stiffness parameter ε we obtained along with general difficulties in attaining a rapid convergence toward physiologically realistic parameter estimates, motivated a re-examination of the general problem of the ML estimation of our model. We have successful implemented a state-of-the-art ML fitting procedure based on a genetic algorithm. It has addressed some of the computational difficulties described above.

Finding global optima of complex functions containing local minima and/or flat regions is an active area of numerical analysis research because there are no universally accepted methods to solve function optimization problems. One of the most promising classes of algorithms that have arisen to deal with these issues are genetic algorithms (GA), in particular the differential evolution (DE) algorithm (Storn and Price, 1995; Storn and Price, 1996; Price and Storn 1997). Unlike commonly used GA algorithms; the DE algorithm contains only two control parameters, requires no function derivative calculations, requires no special binary encoding of the data, can be applied to most classes of functions, and can handle problems of very high dimensionality. The DE algorithm may be considered as a stochastic optimization procedure that starts with a random population of trial solutions, and then establishes a survival-of-the-fittest competition between this population and another generated by random mixing of trial solution vectors. Iterative application of this method leads to a contraction (i.e. evolution) of the trial solutions onto optimal likelihood function regions. The robustness of the DE algorithm obviates our previous decomposition of the parameter set into linear and nonlinear sets and as a consequence, the need to solve any linear system. That is, we possibly eliminate an important source of numerical instability in our problem. *We have tested this hypothesis by successfully developing an object oriented Fortran 95 code to carry out maximum likelihood estimation using*

a DE genetic algorithm applied to simulated core-temperature data based on the Kronauer model.

Findings from the Application of the Genetic Algorithm. Not only did this new algorithm/code implementation exhibit markedly improved convergence properties, but it also allowed quantification of suspected statistical indeterminacies in the original Kronauer model parameter estimation. Further simulations yielded a variation of the van der Pol model with reduced parameter overdeterminacies. Numerical simulation of a 28 hour forced desynchrony experiment helped us understand that the stiffness parameter ε could be estimated only with great difficulty due to the rapid decay of dynamical transient information crucial to the estimation of this parameter. The minimal curvature (flatness) of the likelihood function with changes in ε caused numerical problems for the quasi-Newton ML algorithm. DE is less sensitive to this problem; it identified it and allowed accurate estimation of the remaining parameters. *Therefore, for our data analyses, we fix the value of this parameter as well in the model fitting.*

Our simulation analyses identified a second indeterminacy arising from the presence of the circadian modulation parameter m in the light driving term of Eq. 8. Simulations show that its influence on the Kronauer model solution is sufficiently weak that it may be neglected. That is, if the ambient light levels are low, we may fix $m=0$ and still estimate the other parameters accurately even if the simulated data were constructed with the value $m=0.667$ suggested by Kronauer. *Since this circadian modulation parameter appears to be arbitrary for simulations conducted here, we will fix it to the value used by Kronauer who found that it could play an important role in his phase resetting work.*

We also compared model fits that took into account the AR(1) structure of the additive noise with standard least square estimation which assumes uncorrelated Gaussian additive noise. Our results suggest that omitting the noise correlation leads to a significant overestimation of the magnitude of the proportionality constants between the light level and the light driving strength in Eq.8. *This result supports the importance of our general approach of emphasizing accurate representation of correlations contained in the thermoregulatory fluctuations and measurement noise.*

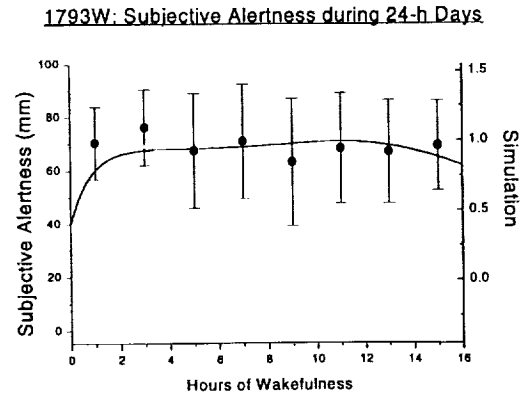


Figure 1. Subjective Alertness averaged over each waking hour of the 25 days of the 24 hour protocol (dots with standard error bars). Solid curve is the model prediction from the Jewett Subjective Alertness Model.

Our results as well as those of Klerman et al. (1996) suggest that for the experiments conducted in Years 1 and 2, the ambient light intensities are sufficiently low (< 15 lux) that the light driving term may be neglected in the Kronauer model with little consequence for the purpose of fitting the equation to experimental data. This approximation removes the problem of

estimating the light driving term with only a negligible overestimation of the magnitude of evoked effect. Estimating the light driving effects in low lux levels becomes mission critical during the experiments conducted in Year 3. In these experiments the effect of circadian phase resetting due to specific light pulse patterns will be determined. These light pulses introduce an additional time scale (~ 12 to 48 minutes) associated with light driving which is appreciably different from the time scale of the evoked effect (28 hours). The light driving will be unmasked from the evoked effect in a manner similar to how the forced desynchrony unmasks the base-line circadian oscillation from the evoked temperature component.

C. Analysis of Performance and Subjective Alertness

The neurobehavioral model developed by Dr. Jewett for both the cognitive performance and the subjective alertness assume that each of these processes may be modeled as the sum of three separate contributions: a homeostatic component, a circadian contribution, and a sleep inertia function. The forms of the cognitive performance components are similar to their subjective alertness counterparts however, the numerical values of their model parameters differ. All functions have an explicit dependence on the instantaneous values of the circadian state variables in the Kronauer model and the subject's recent sleep-wake history. Given the state variable inputs from the Kronauer model, subjective alertness and cognitive performance are computed by solving the linear differential equations for the three components.

In order to implement the neurobehavioral models for analysis of the protocols in **Project 1**, simulations studies were conducted using the original Jewett neurobehavioral models that uses the output of our mathematical model of the effects of bright light on the circadian pacemaker to determine circadian phase. From this preliminary analysis, it was clear that the original neurobehavioral models did a reasonable job of predicting the neurobehavioral data from the subjects in **Project 1** and that no changes in scaling factors or parameters of the neurobehavioral portion of the models were necessary. We next used circadian phase information obtained directly from the subjects' core-temperature series as described above to provide input to the neurobehavioral model. Once the circadian portion of the core-temperature was derived for each subject, an appropriate scaling factor was determined for that subject to normalize the amplitude of the circadian component. The neurobehavioral models were then refined in order to incorporate the circadian component determined directly from each subject's core-temperature data as described in **III A**. Finally, predictions were made of subjective alertness and cognitive throughput using the output of these

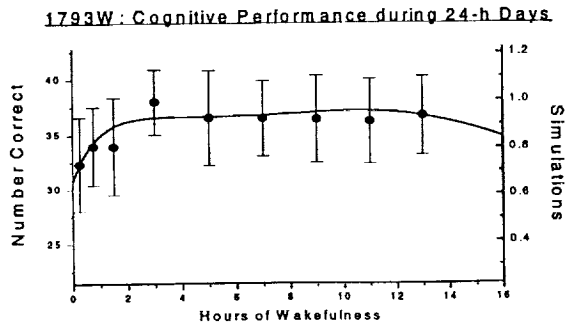


Figure 2. Cognitive Performance averaged over each waking hour of the 25 days of the 24 hour protocol (dots with standard error bars). Solid curve is the model prediction from the Jewett Cognitive Performance Model.

refined neurobehavioral models. The refined models provided an excellent fit to the subjects' average neurobehavioral data during both 24-h entrained days and 28-h forced desynchrony days. Examples of these fits are shown in Figs. 1 and 2. *We now have an established protocol for providing circadian input extracted from experimental data to the neurobehavioral models and then for comparing each subject's average performance on the neurobehavioral test battery with the model predictions. We can conduct a similar analysis for subjective alertness.*

In order to facilitate efficient prediction for our future analyses, much effort was placed into development of a set of computer codes written in Matlab and C with user-friendly graphical user interfaces. This software will henceforth permit prompt analysis of neurobehavioral and circadian data with the neurobehavioral model.

We completing analysis of subjective alertness and performance for all of the subjects in the control group **Project 1**. Since most of the 24-hour experiments resulted in entrained circadian dynamics, we invested a greater effort in Year 3 into analyzing performance/alertness during the forced desynchrony protocol. This will allowed us to study our model predictions of performance and subjective alertness at adverse circadian phases.

D. GROWTH HORMONE MODEL

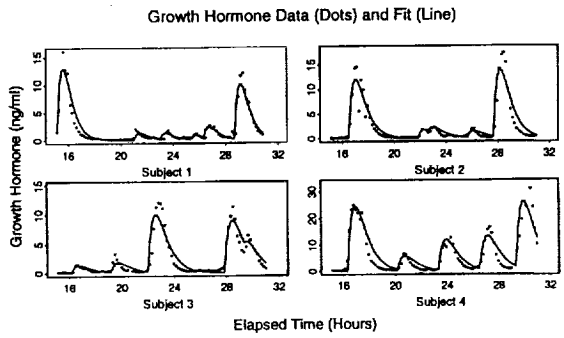


Figure 3. Fit (solid line) of a two-dimensional linear ordinary differential equation model to growth hormone (dots) in 4 healthy subjects observed on constant routines.

Because of the growing interest in the use of growth hormone as a marker rhythm for the human circadian and sleep wake systems, we have developed a two-dimensional linear differential equation model of growth hormone plasma levels on subjects during scheduled days and the constant routine. The model includes a feedback term to measure the effect of growth hormone plasma levels on its on secretory regulation. The model is fit to experimental data by maximum likelihood and has been successfully used to analyze growth hormone data from normal women and ones with fibromyalgia as part of a collaboration with Dr. Gail Adler at the Brigham and Women's Hospital. An example of the model fit is given in Fig. 3. A manuscript on this work is in preparation. *These results are critical to our work on this project because they define an approach to algorithm design that may be used to estimate accurately circadian phase, amplitude and period information from the long melatonin series collected on the 25 24 hour day protocol and the forced desynchrony protocol.*

E. CORTISOL MODEL

We have also submitted for publication a manuscript detailing a new stochastic differential equation model of plasma cortisol levels. This model may be used to analyze diurnal cortisol patterns as well as serving as a starting point for extending our work on growth hormone to the analysis of melatonin series with more than one secretory event.

F. IMPLICATIONS FOR FUTURE RESEARCH

We have developed more accurate statistical models of human circadian marker rhythms including core-temperature, growth hormone and cortisol. These new models provide an accurate means of assessing characterizing circadian physiology with respect to standard marker rhythms in both space and non-space related research. We have used the dynamic phase information from the core-temperature model as an input to the Jewett performance and subjective alertness models. The paradigm may be useful for developing accurate strategies to monitor the circadian health, neurobehavioral state of astronauts during long-duration space missions and for implementing and measuring the effects of countermeasures when significant alterations in these states are detected.

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National Space Biomedical Research Institute

Final Project Report:

Ground-Based Study and Evaluation of Principal Investigator-in-a-Box *

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Ground-Based Study and Evaluation of Principal Investigator-in-a-Box

EXECUTIVE SUMMARY (FY2000)

The efficiency of crew performance on the ISS will depend critically on training and coaching, both before launch and during a flight increment. This project investigated the ability of a real-time expert system to improve performance and reduce the time needed to diagnose errors and troubleshoot a space life science experiment. The experiment tested the efficacy of *Principal Investigator-in-a-Box*, or [PI], a tool for assisting relatively untrained "astronaut surrogates" to detect, diagnose, and correct realistic instrumentation anomalies. It is the first evaluation of such a decision aid under controlled conditions, and will help determine the applicability of expert systems in future space flight research. Two groups of subjects, receiving identical training on sleep monitoring, were tested on two days. Half the subjects were tested with [PI] assistance only on Day 1; the other half only on Day 2. For all subjects, time to detect and identify randomly introduced artifacts and to complete a normal sleep monitoring calibration was measured with and without [PI]. The expert system rules build on the existing [PI] software for troubleshooting and error detection developed for the Neurolab sleep experiment.

Key Findings

Results from the study indicate that an expert system can be used for fault management in a space life science experiment. Furthermore, astronauts who used [PI] during missions found it to be a useful decision aid [5]. However, its utility depends, at least in part, on training and the user's computer literacy.

The feasibility of the PI-in-a-Box concept has been shown in ground studies as well, confirming the favorable experience with it in space. With appropriate training, [PI] reduced time to detect and time to correctly troubleshoot faults in a sleep instrumentation setup. We found that by observing the reliability of the indicator lights, [PI] was helpful for subjects on Day 1, and was a hindrance for them on Day 2. There were also fewer undetected anomalies and undiagnosed faults with [PI] than without it [2,5,6].

Satisfaction of Hypotheses

As stated in the proposal, our hypothesis is that use of a computer decision aid (PI-in-a-Box) will improve experiment performance on three independent measures, compared to the same subjects' performance without the decision aid. These independent measures are:

1. Average time to detect the deterioration of signal quality to beyond a pre-determined level,
2. Average time to identify correctly the source of unanalyzable data in a complex situation with several alternative causes,
3. Average time to complete a normal calibration and run a physiological experiment.

The pilot study and observations of Neurolab and STS-95 data were used to evaluate [PI]'s ability to help with detection times for anomalous signals. The results of the pilot study showed that [PI] assistance reduced the detection time, though not by a statistically significant amount. Training, or the cross effect of [PI] and Day, was found to be significant. The study also found that the number of undetected anomalies was significantly lower when [PI] was available. Gender effects were also found to be significant for the detection task.

The Neurolab and STS-95 data were comprised of signal recordings of the first few minutes of each instrumentation session. It was found that [PI] correctly detected 84% of the anomalies that were not saturated in the signals from the Neurolab data [5]. In the STS-95 data, [PI] correctly detected 86% of all signal anomalies [see section 5 of Appendix A]. Overall, the cardiorespiratory indicator lights were the most reliable, while the electroencephalogram (EEG), and electro-oculogram (EOG) signals were the most prone to false alarms from [PI] indicator lights.

The study completed in Phase 1 showed that the use of [PI] assistance has a different impact for different types of stimulus files. It seems to neutralize differences between signal anomalies of different simulation files. Furthermore, in Phase 2, subjects allowed fewer faults to go undiagnosed (i.e. fewer timeouts) when [PI] help was available. These are positive indications that [PI] acts so as to make complex faults easier to detect and diagnose.

Phase 2 of the study demonstrated a beneficial effect of [PI] and training in reducing anomaly troubleshooting time. Questionnaires showed that most subjects preferred monitoring the [PI] indicator lights *while* monitoring waveforms, rather than monitoring the waveforms alone. On one hand, [PI] did not improve the reliability of detection, since subjects were not any more correct in their anomaly detection with [PI] than without it. On the other hand [PI] did even out performance by reducing the chance of an undiagnosed fault, and by helping subjects with different tasks based on their experience level. It was shown that [PI]'s indicator lights only needed to be 40% reliable for subjects to achieve optimum performance, which shows its flexibility. [PI] correctly detected the anomalous signal for up to 85% of the time [2]. There was no difference in fault management performance between genders.

Implications of Results

Our space experiences with computer decision aids for astronaut scientists have all been demonstrations, rather than formal experiments with testable hypotheses. The drive to develop useable new technology in feasible, cost-effective ways outweighed the scientific need to fly placebo devices as controls for experiments. (These devices would have contributed nothing to the ongoing experiments, would have consumed valuable space resources, and so were considered unessential/dispensable.) Our study performed thorough ground tests to evaluate the efficacy of our expert system for assisting astronauts in the Space Station era. The diagnostic aids, experimental scheduler, and interesting data monitor were shown to be beneficial for carrying out space experiments. Each of the tools developed throughout the history of [PI] – from STS-40 and STS-58 through STS-90 and STS-95 - can be applied to experiments aboard ISS. Autonomous systems are already being implemented in the ISS, and having a software with embedded knowledge such as [PI] will ensure the scientific and operational success of a mission.

These developments could reduce the chance of error caused by human-system interface problems, a concern outlined in section 6.09 of NASA's Critical Path Roadmap.

The development of [PI] can be applied to earth-based domains too. Subjects could be helped by an intelligent fault management system for diagnostics and repairs. Earth-based space research includes projects such as the BIOPLEX. Autonomous fault management systems are already being used for this testbed of life support systems. The results of human behavior in a fault management situation, such as in this ground study, could lead to better designs for the interface of such systems. Other earth-based applications include home sleep monitoring. Patients or caregivers who are not familiar with sleep instrumentation can use a diagnostic engine to help them detect and repair failures, without data being lost. Therefore, the concept of embedding the knowledge in an autonomous system in the spirit of [PI] can benefit technology on earth.

PROJECT RESEARCH ACTIVITY

The expert system [PI] was designed to assist astronauts or other operators in performing experiments outside their field of expertise. The first version of [PI], also known as the Astronaut Science Advisor (ASA), is the first documented attempt to use a biomedical diagnostic expert system on a space mission [11]. [PI] was used to assist astronauts in the performance of the Rotating Dome Visual-Vestibular Interaction Experiment on the STS-58 Space Life Sciences 2 (SLS-2) Space Shuttle mission in 1993 [11]. This first version of [PI] provided data collection capabilities, as well as protocol assistance, scheduling, and protocol modification suggestions. An additional feature consisted of an "interesting data" filter, designed to perform quick-look data analysis and report any unexpected findings to the astronauts during the experiment. Although crew feedback on this demonstration was positive, no data was taken concerning the performance of [PI] or the correctness of the advisories that it issued.

Building upon our past success, [PI] was adapted to help the crew calibrate instruments in flight for a Sleep and Respiration Experiment that flew on the Space Shuttles STS-90 and STS-95 [5]. [PI] displayed electrophysiological signals in real time, alerted astronauts via light emitting diodes (indicator lights) when poor signal quality was detected, and advised astronauts how to restore good signal quality.

Hypotheses

Use of PI-in-a-Box will improve experiment performance on three independent measures, compared to the same subjects' performance without the decision aid. The independent measures are:

1. Average time to detect the deterioration of signal quality to beyond a pre-determined level,
2. Average time to identify correctly the source of unanalyzable data in a complex situation with several alternative causes,
3. Average time to complete a normal calibration and run a physiological experiment.

Research Design

In order to test these hypotheses, MIT undergraduate and graduate students were recruited to act as astronaut surrogates in running the Neurolab sleep experiment. Two separate and increasingly interactive experiments were used to test [PI] rules during the 28 months of this project.

Phase I

During this phase of the study, the time for test subjects to detect and identify the nature of a signal abnormality was tested. In preparation for the experiment, we augmented our library of nominal and abnormal sleep data to represent all known variations of single-channel and multiple-channel failures. As in Smith's 1997 research [10], a BWH technician helped us to produce each of the signal failures. Unfortunately, this method was unsuccessful (For reasons outlined in the NSBRI Project Year 1 summary), and instead two separate files that were recorded during spaceflight were played back for the Phase One experiment. The signals were processed through the Vitaport2 digital sleep recording system and stored on the FlashRAM data card; they were played back for the test subjects through the ThinkPad laptop computer, which either enabled or disabled the [PI] diagnostic routines. Half the thirty-two students, none of whom had previously been exposed to human sleep research, began with 1.5 hours of standard technician training before attempting to monitor sleep physiological parameters from two subjects. The ThinkPad received test subject inputs for detection and diagnosis of signal failures, and made the time and accuracy data available for later analysis.

Phase 2

Phase 2 extended the investigation with thirty-two new subjects working under the supervision of a trained sleep operator (MIT staff, trained by BWH) to evaluate the troubleshooting assistance afforded by [PI]. The pilot study in January 1998 indicated a gender effect in use of [PI] for detecting faults. For this reason, equal numbers of males and females were used to determine if any gender effects occur. During this portion of the project, the test subjects not only detected signal problems, but also interacted with the sleep subject via the computer and the trained operator, to complete the diagnosis and repair. Once again, a balanced crossover design was used, and time and accuracy of the troubleshooting were measured.

Fault Tree Analysis and Diagnostics Development

Before the NSBRI grant was awarded, a three-stage process for developing the PI-in-a-Box diagnostics was completed under NASA Ames Research Center grant number NCC 2-570. A fault tree analysis was performed in order to identify all known failure modes of the system [10]. Using the sleep research experiment hardware and software, each of these failure events was simulated in order to determine the system response. After each failure simulation, the corrective steps required to return the system to its nominal operating state were recorded. Based on these corrective steps, a diagnostic diagram was developed for each system state. These diagnostic diagrams, which were modeled after the malfunction procedure diagrams flown on

each NASA space shuttle mission, served as the basis for the development of the PI-in-a-Box diagnostics used in flight and for the NSBRI funded ground-based studies. Results from the fault tree analysis and diagnostics development culminated in Robin Smith's unpublished Master's thesis [10].

January 1998 Pilot Study

Goals of the January 1998 pilot study were to:

1. Provide preliminary results to determine the appropriate size and characteristics of the test subject pool for the complete study to follow;
2. Determine the appropriate level of training for the subjects;
3. Determine the specific parameters on which the three-year study will focus, i.e., the conditions under which a real-time expert system such as [PI] is most likely to prove beneficial in the performance of an experiment.

As the first expert system ever designed to be an integral part of a Space Life Sciences experiment, a formal and structured evaluation of the efficacy of such a system was unprecedented. The pilot study was a preliminary assessment of the efficacy of [PI] with the "poor signal quality detection" process. Twelve subjects were required to monitor a set of pre-recorded physiological signals and identify signal artifacts displayed on the screen. Every subject performed the experiment twice, once with the assistance of [PI] and once without, in a balanced design.

Results indicated a positive effect of [PI] on overall time to detect anomalies [5,6]. The combination of previous exposure to signal monitoring (training) and [PI] assistance was a significant factor in the improvement of overall reaction time (see section 1 of Appendix A). Also, the assistance of the expert system dramatically reduced the number of undetected anomalies. A significant gender effect was also observed in the data, with female subjects performing better overall compared to male subjects.

Since [PI] had been designed for a life sciences experiment, its evaluation was modeled after that of ground-based medical information systems. As for most medical expert systems, evaluation is an iterative process, and this study represents the first step, providing many insights and recommendations for more in-depth studies in the future, as well as exploring possible ramifications and expansions of the uses of expert systems in space. Results of the pilot study are described in detail in Luca Callini's unpublished Master's thesis [6] as well as in the *Aviation Space and Environmental Medicine* (*ASEM*) publication [5].

STS-90 (Neurolab)

Principal Investigator-in-a-Box was flown with the Neurolab Sleep and Respiration Experiment, launched on the Space Shuttle Columbia on April 17, 1998. [PI] displayed physiological signals in real time during the pre-sleep instrumentation period, alerted the astronauts when a poor signal quality was detected, and displayed steps to improve quality. The

diagnostics developed under the NASA Ames grant were included in this flight version of the software.

Analysis of the post-flight data gathered from the Neurolab Mission was performed [5]. After replaying the physiological signals on the ground, the frequency of correct [PI] alerts and false alarms (i.e., incorrect diagnoses by the expert system) was determined in order to assess the robustness and accuracy of the rules. For the in-flight performance, excluding the saturated signals, the expert system had an 84% detection accuracy, and the questionnaires filled out by the astronauts showed positive crew reactions to the expert system. Results from the Neurolab Sleep Experiment are described in detail in the *Aviation Space and Environmental Medicine (ASEM)* publication [5].

A summary of the results of this analysis is provided in section 4 of Appendix A.

STS-95

In order to quantify the performance of the [PI] expert system in conjunction with the Sleep 2 experiment, the first 15 minutes of sleep data from each of the eight files were extracted and analyzed. An MIT graduate research assistant trained by BWH technicians reviewed each of these 15-minute files several times. Every time [PI] displayed a red state light, indicating poor signal quality, the research assistant judged whether the signal quality indication was correct and further diagnosed the general signal anomaly as flat, saturated, noisy or popping. It was found that [PI] detected an anomalous signal 86% of the time it indicated a poor signal.

A summary of the results of this analysis is provided in section 5 of Appendix A.

The Ground-Based Study

Phase 1

The first phase of the experiment tests the efficacy of [PI] for assisting "astronaut surrogates" in detecting realistic experiment artifacts in the context of a space life sciences experiment that monitors sleep. For this initial phase of the study, matched groups of subjects were tested, each receiving identical training on sleep monitoring. The time for test subjects to detect and identify the nature of a signal abnormality was measured. Pre-recorded electrophysiological signals were played back for the subjects through a PC laptop computer, which either enabled or disabled the [PI] diagnostic routines. The subjects, none of whom had previously been exposed to human sleep research or [PI], began with 90 minutes of training before attempting to monitor electrophysiological sleep parameters. Half the subjects received [PI] assistance in their first exposure to the tests, and half in their second. The laptop recorded test subject inputs for detection and diagnosis of signal failures, and made the time and accuracy data available for later analysis.

Two distinct fault stimulus files were used for the analysis, labeled File A and File B. Without [PI] subjects found it more difficult to correctly identify saturated and noise signals in file A than they did in file B. Consequently, [PI] was more beneficial when used with file A, as

indicated by the significant cross effect of [PI] X file on signal identification for saturated and noise signals. Furthermore, [PI] had a significant effect on improving subjects' ability to correctly identify saturated signals in file A. Though subjects found file A much harder to interpret than file B when [PI] was not active, the difficulty of the files became similar when [PI] was active. This result is encouraging. It suggests that any peculiar differences between files, which might affect subject performance for correct identification of anomalies, are essentially nullified when [PI] is active. [PI] appears to be more effective in situations that subjects find difficult—but this result is fairly intuitive.

Regarding the detection of anomalies, a statistically significant effect of file was observed for saturated signals. A cross effect of [PI] X File was also observed for the detection of popping signals, indicating that subjects found it much harder to detect popping signals in file B than in file A. Consequently, [PI] was more beneficial in assisting with detection of popping signals in file B.

A statistically significant effect of day on response time was observed for popping signals. No other statistically significant effects on response time were found. This is consistent with the pilot study, which found only a significant effect of [PI] X Day on response time for saturated signals. Phase 1 analysis is provided in section 2 of Appendix A.

Phase 2

Phase 2 of the ground-based study sought to assess the utility of on-board expert systems, in general, for performing experiments and troubleshooting complex instrumentation systems. Results from this study can be applied to nuclear power plant control rooms, airplane cockpits, or wherever complex human-computer interaction is present. Thirty subjects, divided into two groups, received training on the sleep instrumentation and the [PI] interface. Each subject was then tested on two separate days with a subject instrumented in real time. Group 1 received [PI] help only on Day 1; Group 2 received [PI] assistance only on Day 2.

Results indicate a beneficial effect of [PI] and training in reducing anomaly troubleshooting time. However, only training was found to significantly reduce detection time. In addition, no significant gender effects were found in this study. Post-experiment questionnaires showed that most subjects preferred monitoring the [PI] indicator lights *while* monitoring waveforms, rather than monitoring the waveforms alone. [PI] indicator lights were correct up to 85% of the time for single-channel faults. But [PI] did not improve the subjects' reliability, since they were not any more correct in their ability to detect anomalies with [PI] than without it. But [PI] acted as a regulator of fault management. One way is by reducing the chance of a timeout while diagnosing a fault. Another way is by reducing the planning time for diagnostic search and fault detection when subjects were inexperienced on Day 1, but reducing the time for executing the task of asking questions when subjects had some experience with the system on Day 2. Helping subjects with different tasks based on their experience level shows that [PI] can regulate overall fault management performance. A summary of Phase 2 data can be found in section 3 of Appendix A.

Subjects were also shown to correctly interpret [PI] indicator lights despite the presence of false positives. Each experimental group showed fundamental differences in the way they learned the instrumentation and proceeded with fault management. Results from this study have been presented at the SmartSystems 2000 conference [3], and in Allen Atamer's and Mindy Delaney's unpublished Master's theses [2,7]. In addition, a publication in the journal of the Human Factors Society is expected to be published sometime next year [1].

IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

Impact of research on future space biomedical research

Building on past experience with the rotating dome (STS-58) and sleep experiments (Neurolab/STS-90, STS-95), [PI] could be adapted for use aboard both the Space Shuttle and the International Space Station (ISS) to help astronauts carry out other experiments. [PI], designed as an integral part of a sleep monitoring experiment, assisted the Neurolab astronauts with the calibration and troubleshooting of the instrumentation during the pre-sleep period of the experiment. During this time, mission rules precluded investigator ground-to-air contact with the crew. Because the crucial experiment setup and calibration for the extended period of sleep monitoring was performed during the no-contact phase, the crew was necessarily isolated from the true science experts. During this phase of the experiment, [PI]'s role was to display the subjects' physiological signals, identify anomalous signals, and suggest corrective procedures when necessary. In the same way, [PI] could be extended to other applications for experiment/scientific support.

The [PI] interface could alleviate some of the human-system interface problems astronauts will encounter on the ISS, as described in section 6.09 of the NASA Critical Path Roadmap. Although highly intelligent, well-educated and versatile, most astronauts will inevitably face the need to execute an experiment outside their field of expertise. [PI] can offer the right knowledge for astronauts to use: diagnostic aids, an experimental planner/scheduler, and an interesting data filter. The ability of the human-*autonomous agent* team to carry out expert decision-making in an isolated, confined environment will ensure that both scientific and mission objectives are reached.

Autonomous systems will be implemented into several applications in the ISS operation [8]. For instance, Node 3, to be launched in 2002, will be a connector for several of the U.S. modules. Vigilance monitoring for each of the 8 subsystems of the life support system will be prohibitively time consuming for station and ground crew. 3T, an autonomous software package developed at NASA, will run this life support system. The Remote Manipulator System Assistant (RMSA) will serve to automate the procedures relating to the shuttle's remote manipulator system. There is an equivalent autonomous system for the Space Station Remote Manipulator System (SSRMS) being designed as well. Moreover, the AerCAM, a soccer-ball-shaped free-flying assistant, is also being implemented in station operations. It is designed to inspect the modules for suspicious leaks or faults, and can be inserted in locations that may be risky for the astronaut. Autonomous systems will play an important role in the daily operation of the station.

To ensure the success and effectiveness of a mission, crewmembers must maintain a high level of cognitive performance and vigilance while operating and monitoring sophisticated instrumentation. Astronauts, however, commonly experience stress such as high workload, isolation, and sleep disruption during space flight. Moreover, astronauts aboard the International Space Station (ISS) will nominally have three- to six-month tours of duty. Because it is important for astronauts to maintain high levels of performance throughout long-duration space flight missions, there is a need to develop effective human-machine systems that can overcome these detriments. [PI] offers a promising way of addressing these problems.

Decision aids for fault management

Some of the empirical analysis in the January 2000 study may be referenced as a method of assessing human performance in failure management aboard the ISS. When troubleshooting faults, astronauts may choose to perform different diagnosis tasks than those outlined in the general procedures. Currently this behavior is not particularly endorsed in NASA's operational policy aboard space shuttle. However, with a sophisticated on-going mission such as ISS, flexibility in procedures is important. Both ground and on-board crew need support in interacting with the station, and each other.

Evaluating astronauts' troubleshooting decisions addresses a concern cited in the NASA critical path document (section 6.11). In the January 2000 study, we developed a taxonomy of the types of deviations from the NASA flowcharts made by the astronaut surrogates [2]. Future studies could seek to understand the interaction between a physical model of a system and the model of the system within the astronaut's mind. Predicting human behavior using observables of the system (such as the Bayesian approach used in the January 2000 study) could help us to understand the process by which astronauts decide things within the context of fault detection, isolation and repair.

In the future this knowledge of human fault management behavior can be embedded into a computer assistant that could predict a potentially ineffective path in managing a fault and then alert the astronaut to dead-ends, or a decision based on an incorrect model of the system, etc. This aid would be an effective "coach" for the astronaut in times when there is high workload, fatigue and stress in the ISS environment.

Humans and autonomous systems

Human interaction with the next generation of autonomous diagnostic systems is vital for the success of long-duration missions. Much the way a pilot and Flight Management System coordinate their efforts in an airline cockpit, coordinating between the human and the software agent as a team can be a worthwhile partnership. We should try to avoid the problems observed with current automated systems, such as mode confusion, automation surprises, and other such behavior with sometimes catastrophic consequences. Design of these systems must include properties such as observability and directability to answer the questions "What is going on?" and "Who is in charge?". Ongoing self-analysis and reporting are important features of the agent's functionality. With information provided by the agent, the human can determine what processes should be allocated to the agent, and at what level of autonomy.

Capable of taking over from a human at any level of mission planning and operations, current autonomous agents show great promise in this regard. But their interaction with humans in accomplishing tasks has not been studied and is not well understood. It is essential to open up the agent's insides to inspection, so that feedback from a human can be incorporated into its operating procedures in a way that is understandable by both the human and the agent. Integration of human learning with machine learning can afford a more complete understanding of the systems being monitored. Humans can gather knowledge about a system's behavior that an agent's model of the world may not predict. But an agent is more capable than a human of exhaustively considering all candidate problems with a system-in-fault diagnosis. Each of these parts of the team has different strengths and weaknesses. In pairing the agent's strengths with the human's strengths, we can make mission operations more productive. From the beginning, this has been the long-term goal of [PI] studies.

This push for autonomy in complicated space systems may inspire the same autonomous support for the crew while running experiments on themselves and each other in BIOPLEX. BIOPLEX provides a timely opportunity to develop and test software for interface between crew and autonomous agents in a stand-alone, closed habitat. BIOPLEX should seek to supply tools to enable the crew to maintain and repair the life support systems in a real lunar or Martian habitat. One of these tools should be the implementation of autonomous systems in the everyday operations of such a habitat, including conducting scientific experiments. [PI] was the seminal work for an investigation into implementing autonomous agents for scientific experiments. BIOPLEX provides an appropriate test bed for proving this autonomous agent technology.

Home health care

The appeal of clinical sleep monitoring in home is growing. First, patients tend to sleep better in their own beds than in a hospital. Second, home sleep monitoring costs far less than monitoring done in a laboratory. With current systems of home sleep monitoring, technicians make house calls to instrument the patient with electrodes and setup the equipment. The sleep doctor can then monitor the patient's sleep pattern remotely, by downloading data from the home recording device. This system is generally reliable for home sleep monitoring, and there is tremendous interest in this from the private sector in terms of home health care.

However, there are problems with this scenario that the average patient or caregiver is not equipped to handle. Sometimes electrodes fall off after the technician leaves or during the night. As a result, data is lost or is of poor quality. [PI] as the home sleep monitoring software would detect anomalous signals and suggest ways the patient or caregiver might fix the problem. [PI]'s benefit can be extended from helping untrained astronauts to helping untrained sleep patients or caregivers fix problems with instrumentation. [PI] could be a cost-effective way of improving the reliability of the home sleep monitoring system.

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Appendix A: PROJECT RESEARCH DATA

SECTION 1: JANUARY 1998 PILOT STUDY DATA

TABLE I. MAIN AND CROSS EFFECTS ON AVERAGE REACTION TIMES

Effects	N	T	F	p	Mean Effect (s)
DAY (D)	59	1.337	2.586	0.127	3.322
[PI] Assistance (PI)	59	1.327	2.157	0.161	3.299
PixD	59	1.35	14.953	**0.001**	**10.062**

TABLE II. EFFECTS ON THE NUMBER OF UNDETECTED ANOMALIES

Effects	F	T	p	Mean Effect (anomalies improved)
DAY (D)	1.44	0.805	0.248	4
[PI] Assistance (PI)	4.309	1.93	**0.05**	**9**
PixD	13.196	1.676	**0.002**	**20.333**

SECTION 2A: PHASE 1 DATA (MARCH 1999)

Table I. Analysis of Various Effects on Response Time for Popping Signals

Effect	n	F	P
File	50	0.304	0.584
Day	50	2.362	0.132
[PI]	50	0.346	0.559
[PI] X Day	50	1.162	0.287
[PI] X File	50	0.701	0.407

Table II. Cross Effect of [PI] X Order (Average Fractional Improvement on Signal Detection)

	Pops	Saturation	Noise
File A w/ [PI]	0.17	-0.15	-0.24
File B w/ [PI]	0.18	0.12	0.06

SECTION 2B: PHASE 1 DATA (MARCH 1999)

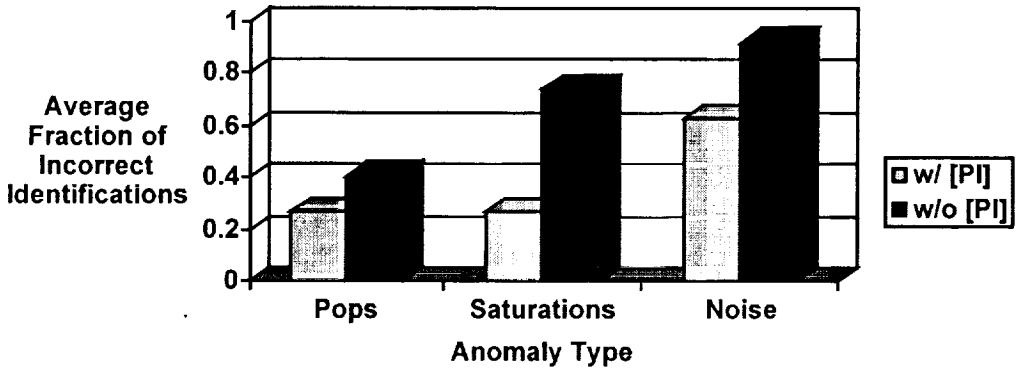


Figure I. Mean Effect of [PI] on File A

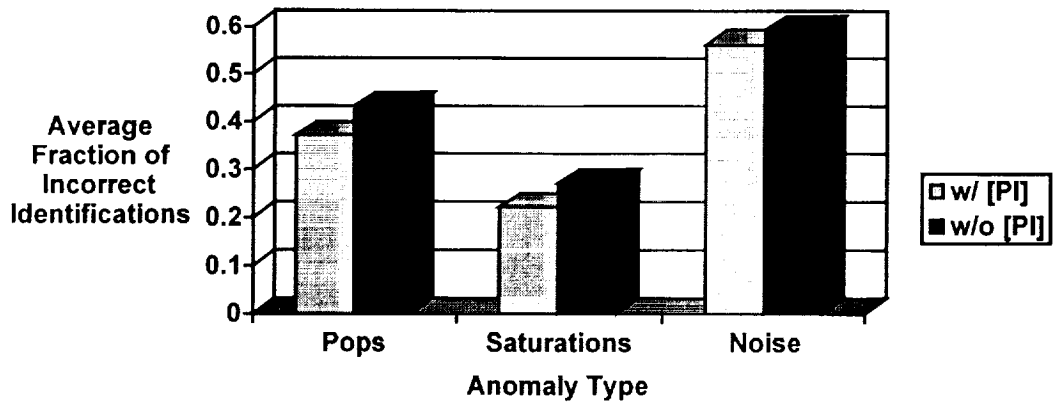


Figure II. Mean Effect of [PI] on File B

SECTION 3: JANUARY 2000 PHASE DATA

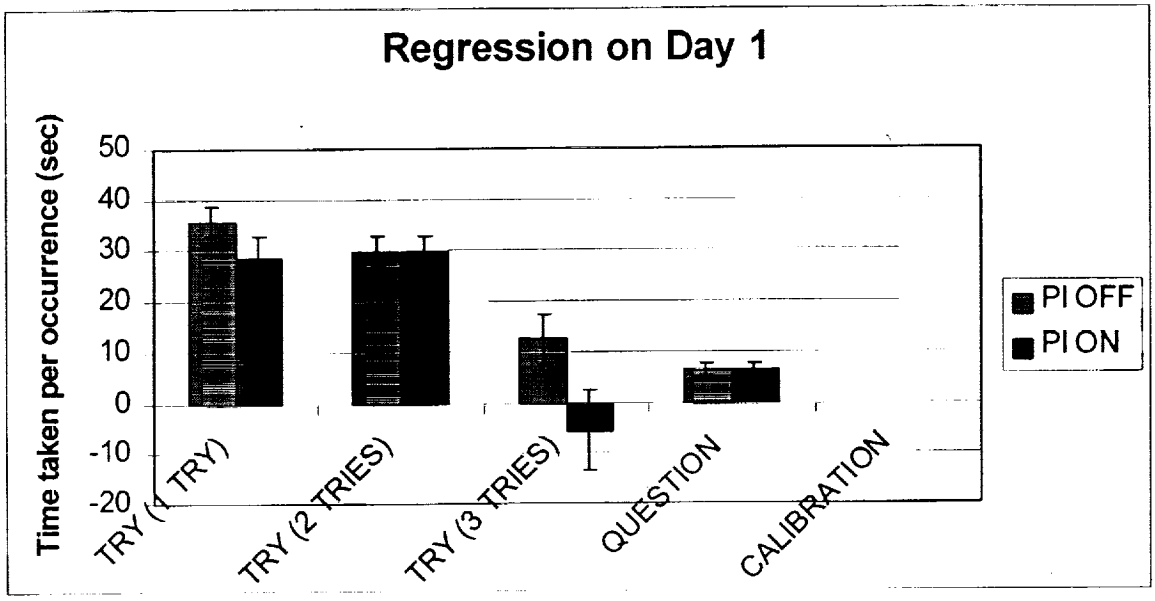


Figure III: REGRESSION OF OVERALL DOWN TIME FOR PLANNING AND EXECUTION TASKS, DAY 1

SECTION 3: JANUARY 2000 PHASE DATA (CONT'D)

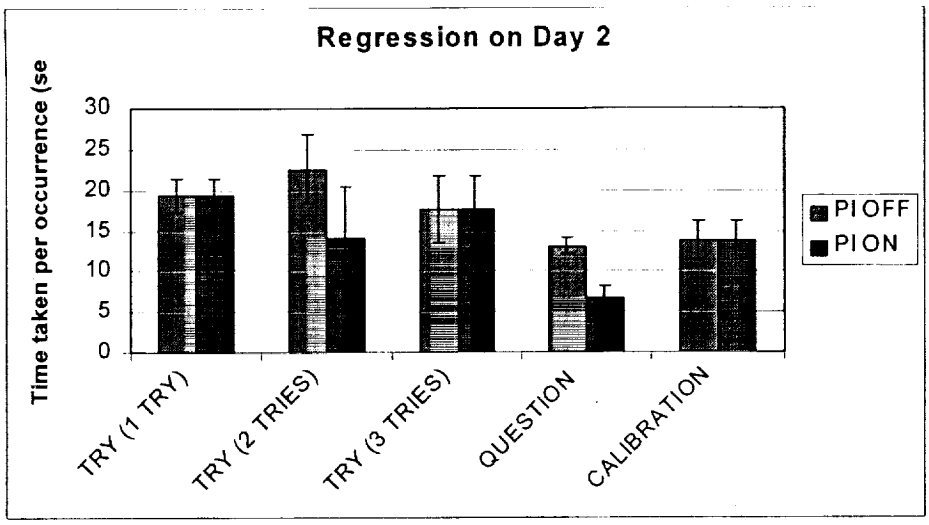


Figure IV: REGRESSION OF OVERALL DOWN TIME FOR PLANNING AND EXECUTION TASKS, DAY 2

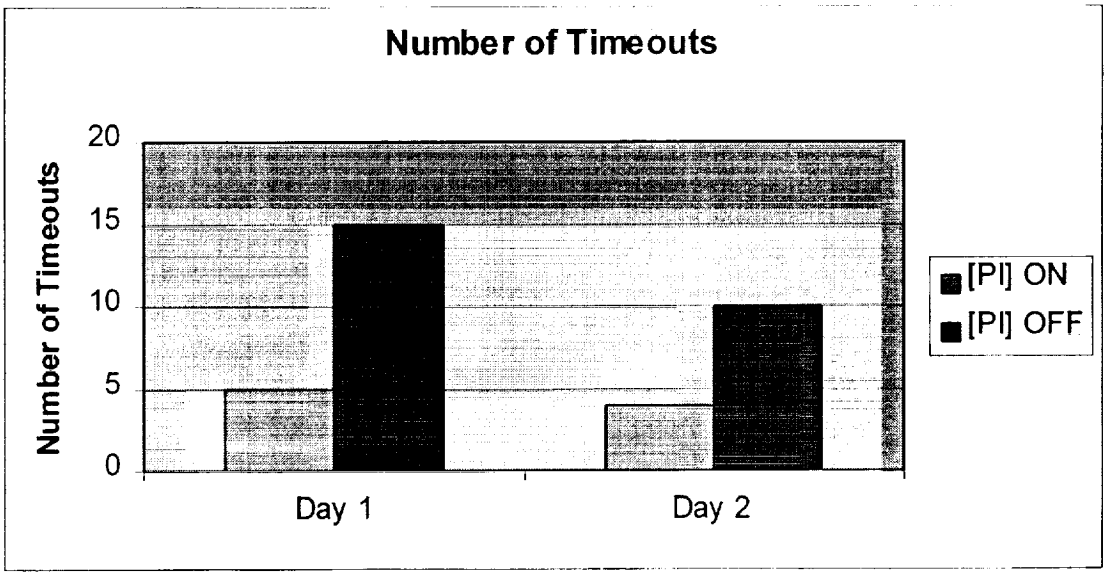


Figure V: Number of timeouts, or undetected anomalies

SECTION 3: JANUARY 2000 PHASE DATA (cont'd)

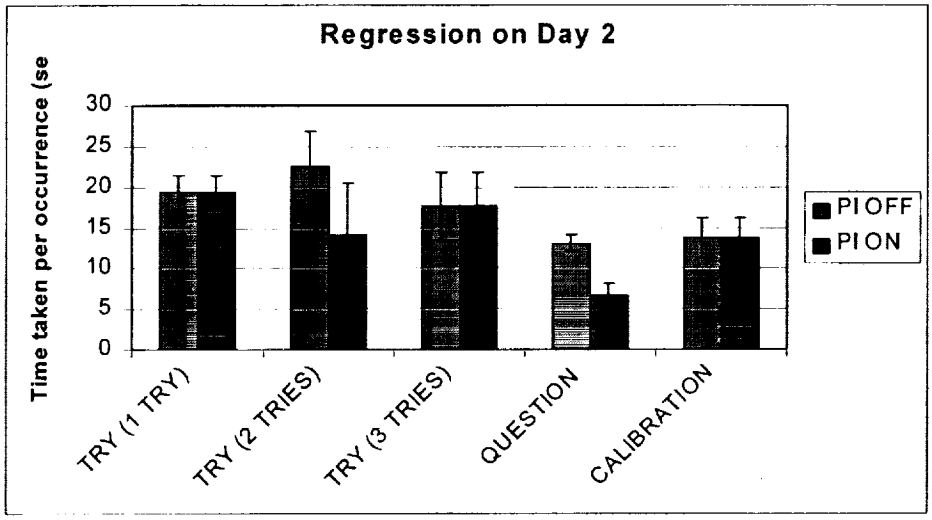


Figure IV: REGRESSION OF OVERALL DOWN TIME FOR PLANNING AND EXECUTION TASKS, DAY 2

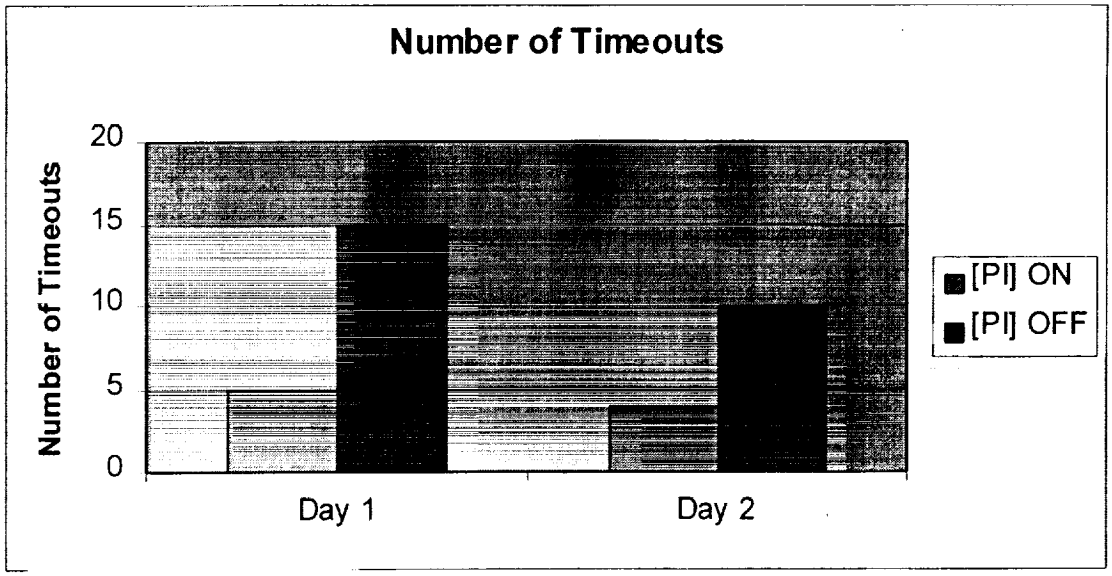


Figure V: Number of timeouts, or undetected anomalies

SECTION 3: JANUARY 2000 PHASE DATA (CONT'D)

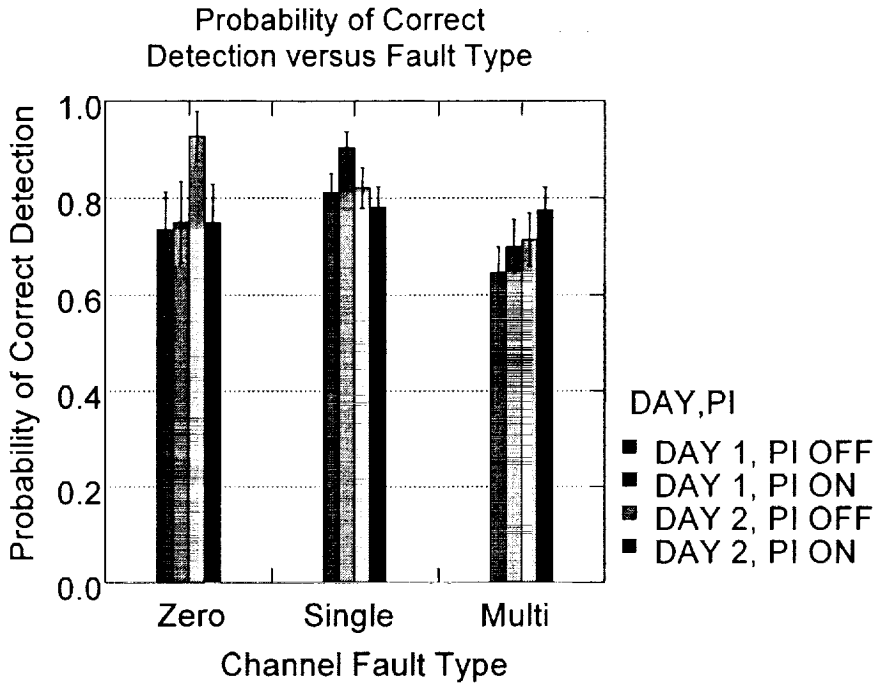


Figure VIII: Percent correct Detection for subjects

SECTION 3: JANUARY 2000 PHASE DATA (CONT'D)

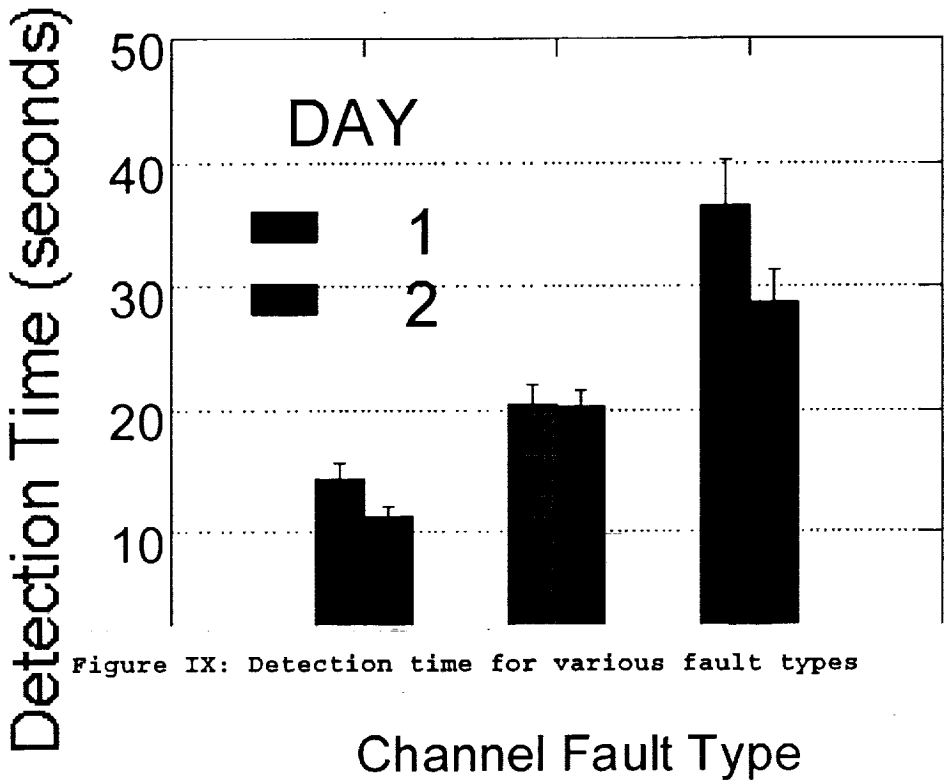


Figure IX: Detection time for various fault types

SECTION 3: JANUARY 2000 PHASE DATA (CONT'D)

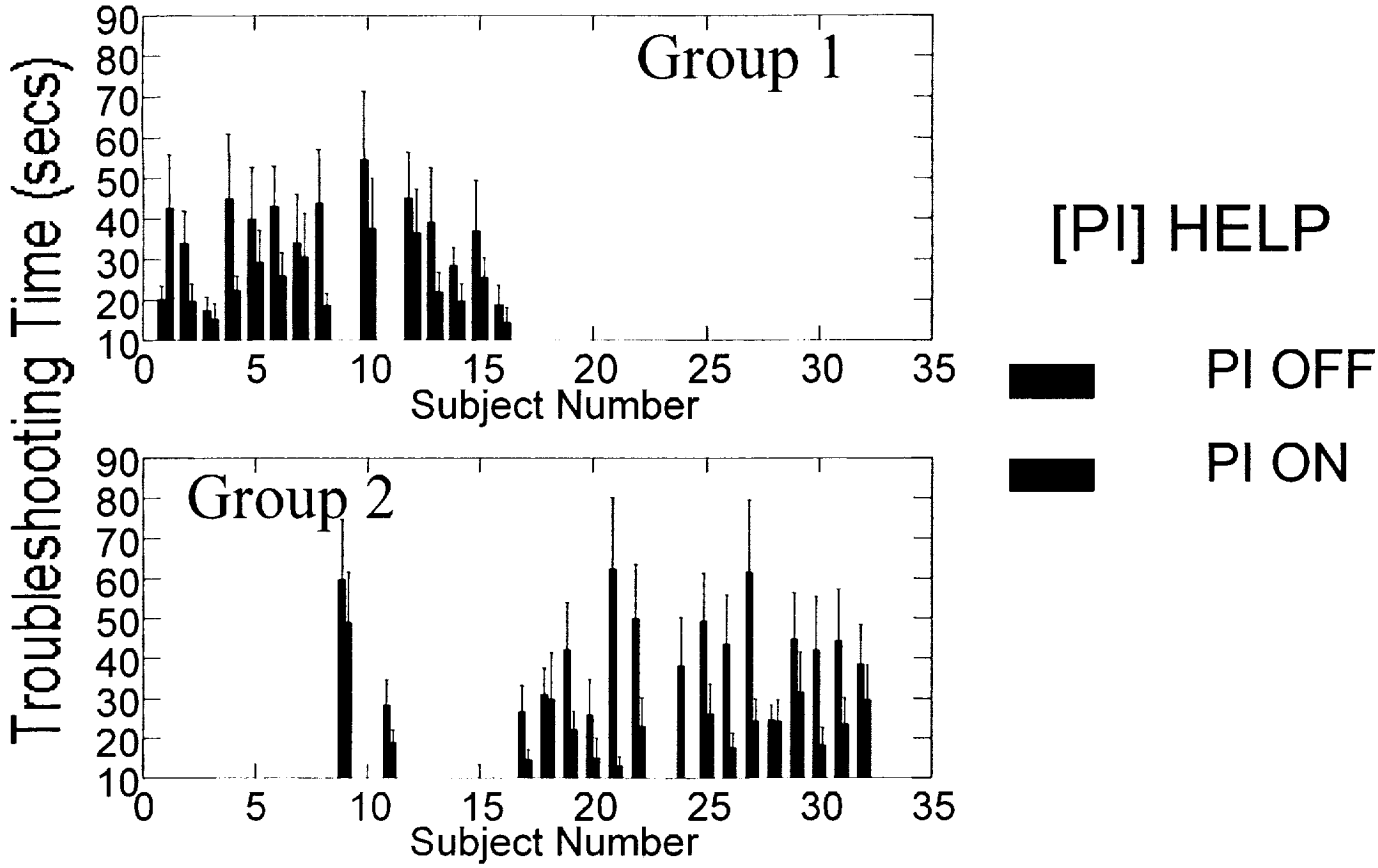


Figure X: Troubleshooting Time for subjects

SECTION 4A: STS-90 DATA

Table III. ELECTROPHYSIOLOGICAL AND CARDIORESPIRATORY SIGNAL IDENTIFICATIONS.

Flight Day	Crew ID	TOTAL EP			TOTAL CR		
		True	False	Saturation	True	False	Saturation
3	1	5	0	0	3	2	0
3	3	2	3	10	21	2	0
4	1	5	0	0	5	5	0
4	3	24	6	5	26	2	0
13	3	11	0	15	21	2	0
13	1	11	0	0	24	0	0
15	4	4	4	5	37	3	0
15	2	2	3	10	24	4	0
12	1	15	2	2	27	3	0
12	3	9	5	11	5	0	0
6	4	24	0	10	9	4	0
6	2	24	5	17	14	2	0
14	4	24	11	9	27	1	0
14	2	9	2	5	15	0	0
5	4	6	1	1	18	5	0
5	2	10	10	17	27	0	0
Total		175	42	100	276	35	0
% Valid with Saturation			55.21%			88.75%	
% Valid without Saturation			80.65%			88.75%	

SECTION 4B: STS-90 DATA

Table IV. OVERALL [PI] FLIGHT PERFORMANCE - NUMBER OF SIGNAL ALERTS.

Flight Day	Crew ID	TOTAL ALL			% VALID	
		True	False	Saturation	w/ Sat	w/out Sat
3	1	8	2	0	80.00%	80.00%
3	3	23	5	10	60.53%	82.14%
4	1	10	5	0	66.67%	66.67%
4	3	50	8	5	79.37%	86.21%
13	3	32	2	15	65.31%	94.12%
13	1	35	0	0	100.00%	100.00%
15	4	41	7	5	77.36%	85.42%
15	2	26	7	10	60.47%	78.79%
12	1	42	5	2	85.71%	89.36%
12	3	14	5	11	46.67%	73.68%
6	4	33	4	10	70.21%	89.19%
6	2	38	7	17	61.29%	84.44%
14	4	51	12	9	70.83%	80.95%
14	2	24	2	5	77.42%	92.31%
5	4	24	6	1	77.42%	80.00%
5	2	37	10	17	57.81%	78.72%
Total		451	77	100	71.95%	84.22%

SECTION 5: STS-95 DATA

Table V. Summary of STS-95 [PI] Performance:

Electrophysiological Signals

File Name	Number of Poor Quality Indications	Number of Correct Poor Indications	Percentage Correct
180a.vpd	123	104	84.6%
180b.vpd	127	99	78.0%
180c.vpd	117	95	81.2%
180d.vpd	69	49	71.0%
180e.vpd	7	1	14.3%
180f.vpd	30	20	66.7%
180g.vpd	5	5	100.0%
180h.vpd	49	30	61.2%
All Files	**527**	**403**	**76.5%**

Table VI. Summary of STS-95 [PI] Performance:

Cardiorespiratory Signals

File Name	Number of Poor Quality Indications	Number of Correct Poor Indications	Percentage Correct
180a.vpd	47	46	97.9%
180b.vpd	43	38	88.4%
180c.vpd	48	46	95.8%
180d.vpd	37	36	97.3%
180e.vpd	62	58	93.5%
180f.vpd	78	78	100.0%
180g.vpd	82	82	100.0%
180h.vpd	84	84	100.0%
All Files	**481**	**468**	**97.3%**

APPENDIX B: LIST OF PROJECT PUBLICATIONS

Atamer, A., "Principal Investigator-in-a-Box Thesis." Unpublished Master's Thesis, Massachusetts Institute of Technology, December 2000.

Atamer, A. and Delaney, M. Effectiveness of Principal Investigator-in-a-Box as an Astronaut Advisor for a Sleep Experiment. Presented at SmartSystems 2000 Conference, Houston, TX, September 6-8, 2000.

Atamer, A. and Delaney, M., Young, L.R. Efficacy of an Expert System for Human Assistance on a Sleep Experiment. Human Factors Journal. Manuscript to be submitted. September 2000.

Atamer, A. and Delaney, M., Young, L.R. Ground Based Study and Evaluation of Principal Investigator in a Box. Presented at National Space Biomedical Research Institute Conference, League City, TX, January 10-13, 2000.

Callini, G. "Assessment of an Expert System for Space Life Sciences: a Preliminary Ground-Based Evaluation of PI-in-a-Box for the Neurolab Sleep and Respiration Experiment." Unpublished Master's Thesis, Massachusetts Institute of Technology, September 1998.

Callini, G., Essig, S.M., Heher, D., & Young, L.R. Effectiveness of an expert system for astronaut assistance on a sleep experiment. Aviation Space and Environmental. Medicine, 71 (9): 1-10.

Delaney, M. "Ground-Based Study of an Expert System for Human Assistance on the STS-95 Sleep and Respiration Experiment." Unpublished Master's Thesis, Massachusetts Institute of Technology, December 2000.

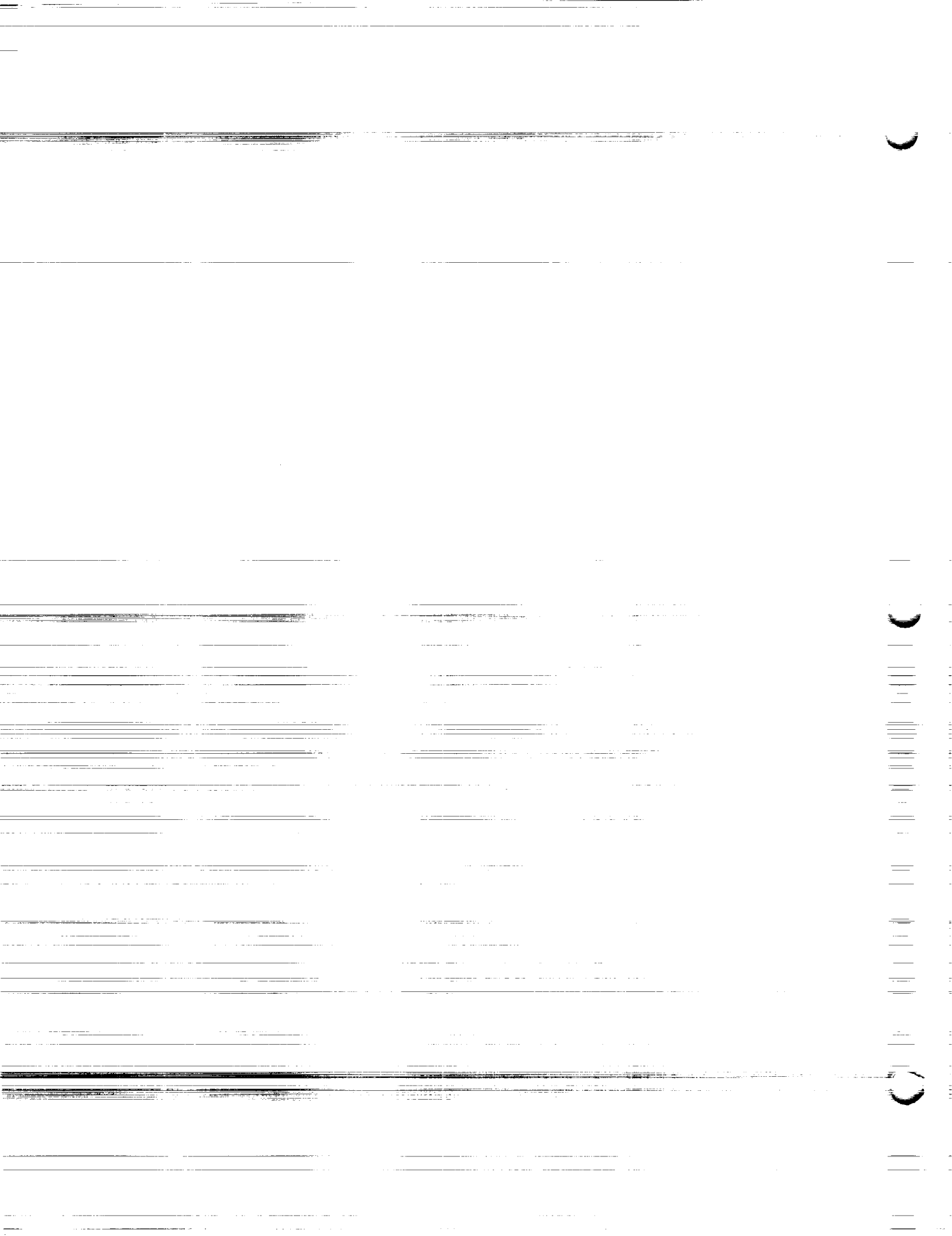
Smith, Robin. "Fault Tree Analysis and Diagnostics Development for PI-in-a-Box with the Neurolab Sleep and Respiration Experiment." Unpublished Master's Thesis, Massachusetts Institute of Technology, 1997.

Young L.R. PI-in-a-Box. *Journal of the Society of Instrument and Control Engineers.* (1994); 33(2):119-22.

NSBRI RESEARCH PROGRAM
IMMUNOLOGY, INFECTION AND HEMATOLOGY

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Shearer, W. T.	**PI**	**Baylor**	**Space Flight Immunodeficiency**
Dinges, D. F.	CO-I	Penn	
Kanwar, S.	CO-I	Baylor	
Lee, B. N.	CO-I	M. D. Anderson	
Lugg, D. J.	CO-I	Australia	
Mullington, J. M.	CO-I	Harvard	
Ochs, H. D.	CO-I	Washington	
Pierson, D. L.	CO-I	NASA JSC	
Reuben, J. M.	CO-I	M. D. Anderson	
Rosenblatt, H. M.	CO-I	Baylor	
Sams, C.	CO-I	NASA JSC	
Smith, C. W.	CO-I	Baylor	
Smith, E. O.	CO-I	Baylor	
Smolen, J. E.	CO-I	Baylor	
Butel, J. S.	**PI**	**Baylor**	**Immune Function and Reactivation of Latent Viruses**
Conner, M. E.	CO-I	Baylor	
Keitel, W. A.	CO-I	Baylor	
Larina, I.	CO-I	Russia	
Lednicky, J. A.	CO-I	Baylor	
Ling, P. D.	CO-I	Baylor	
Lugg, D. J.	CO-I	Australia	
Pierson, D. L.	CO-I	NASA JSC	
Fox, G. E.	**PI**	**U of Houston**	**DNA Probe Design for Preflight and Inflight Microbial Monitoring**
Willson, R. C.	CO-I	U of Houston	
Alfrey, C. P.	**PI**	**Baylor**	**Neocytolysis: Mechanisms and Limitations**
Byrne, B. J.	CO-I	University of Florida	
Rice, L.	CO-I	Baylor	
Trial, J.	CO-I	Baylor	



NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
FINAL PROGRAM REPORT 2000

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William T. Shearer, M.D., Ph.D.
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EXECUTIVE SUMMARY

1. Strengths and Synergy of Team's Research Program

Two of the four National Space Biomedical Research Institute (NSBRI) Immunology, Infection, and Hematology team projects (Space Flight Immunodeficiency, W.T. Shearer, Principal Investigator; Immune Function and Reactivation of Latent Viruses, J.S. Butel, Principal Investigator) are reinforcing and synergistic, in that they both address the same Critical Research Path risks (Immunodeficiency/Infections and Carcinogenesis), and they have actively collaborated in these efforts. These Critical Research Path risks carry a Rank 1 (high priority) and Type III (problem suspected), and these risks have been addressed by both projects. Related to the Shearer and Butel projects is that of G.E. Fox (DNA Probe Design for Pre-Flight and In-Flight Microbial Monitoring) that addresses Critical Research Path Risk of Altered Host-Microbial Interactions (Rank 3, Type III). The purpose of this project is aimed at developing a countermeasures program that will detect bacterial contamination of the spaceship (principally water supply) before it causes infection in astronauts. Somewhat related to the other three projects is that of C.P. Alfrey (Neocytolysis: Mechanisms and Limitations), which examined the possible erythrocyte rupture mechanism as an explanation for the apparent anemia experienced by astronauts as they pass through the stress/shearing force of acceleration and deceleration in leaving and returning to Earth (Critical Research Path Risk of Altered Hemodynamics: Dynamics from Altered Blood Components – Rank 1, Type III).

A real team approach and synergetic results can be easily seen by the efforts of the first two of these four projects (Shearer and Butel). In a sense, these projects are the mirror images of each other. The Shearer project has focused on immune alterations in humans exposed to certain conditions of space travel (stress, isolation, containment, sleep-deprivation, microbial contamination), whereas the Butel project has targeted the identification and quantitation of microbial organisms (principally viruses) in humans exposed to most of the same conditions. In fact, the same subject cohort was used in some of the joint research of these two projects. The common cohorts were two-fold: subjects exposed to the Antarctic winter-over (Dr. Desmond Lugg, Australian National Antarctic Research Expedition [ANARE]) and capsule containment (Dr. Irina Larina, Russian Institute for Biomedical Problems). These two human experiences are thought to mimic some of those that astronauts experience in long-term space travel. Blood, urine, and saliva specimens were obtained from these subjects and initially brought to the National Aeronautics and Space Administration Johnson Space Center in Houston (Dr. Duane Pierson), and then subsequently to the laboratory of Dr. Butel, and finally to Dr. Shearer's laboratory. Plasma specimens for bacteriophage antibodies were sent to the laboratory of Dr. Hans Ochs at the University of Washington in Seattle. In a pre-agreed fashion, specimens were parceled out to each laboratory, where specialized tests of immune function or viral detection and quantitation were performed or planned for the future with stored specimens. The overall plan included provisions for assessing lymphocyte proliferative responses and cytokine production in subjects experiencing reactivation of Epstein-Barr virus, as documented by virus shedding in the saliva and/or DNA probe quantitation of white blood cell pellets. Thus, information on this virus infection in humans exposed to the Antarctic winter or Moscow capsule could be magnified by the team effort of the Butel and Shearer laboratories.

Both Dr. Shearer and Dr. Butel have established synergy projects with other NSBRI investigators involved in sleep deprivation (Dr. David F. Dinges and Dr. Janet M. Mullington – Human Performance, Chronobiology, and Sleep Team). These joint projects were designed to test another condition of space flight (sleep-deprivation) upon the human immune system and its ability to contain viral infections. Studies with Dr. Dinges and Dr. Mullington focused on the production of sleep-inducing cytokines and their receptors and the impact that these altered levels of messengers of the neuroendocrine-immune system might have on host defense. One such consequence would be the upregulation of viral receptors by elevated TH2-type inflammatory cytokines. An example would be the upregulation of CD21, the B-cell receptor for Epstein-Barr virus by high blood levels of interleukin-10. Studies with Dr. Mullington are looking at the shedding of Epstein-Barr virus and JCV, BKV, and SV40 by humans partially deprived of sleep. Additional total sleep-deprivation studies are planned in grant applications to non-NSBRI sources, including the National Institutes of Health ("Earth-Based Research Relevant to the Space Environment", PA-00-088).

Drs. Shearer and Butel have a long-term collaboration in other scientific areas of investigation (e.g., National Institutes of Health Center for AIDS Research – Butel, Director; Shearer, Co-Director). The NSBRI research support has greatly facilitated these interactive project collaborations, because it has expanded the critical mass of investigators, particularly young investigators, in the science of space biomedicine. Drs. Shearer and Butel will be continuing their collaboration in the NSBRI in the next funding cycle, where they will examine an extremely important condition of space flight -- radiation using a murine model infected with latent viruses. For this purpose, they will be collaborating with members of the NSBRI Radiation Safety Team and investigators (Dr. Daila Gridley and Dr. Gregory Nelson) at Loma Linda University, where a linear accelerator is available for generation of the most prominent form of space radiation -- proton radiation, estimated to be 2 Gy in Mars-bound space travelers. These plans will directly address the Critical Research Path risk of carcinogenesis.

In terms of the team collaboration with the two other team projects (those of Fox and Alfrey), there has been more limited interaction. The Fox project has been extremely limited due to a very restricted budget, as recommended by the initial peer review process 3 years ago, and the Alfrey project has been more isolated because of a different emphasis (i.e., cytolysis of new red blood cells). Nevertheless, all four projects have been assisted by the monthly business meeting attendance, conjoint science presentations at national space meetings, and participation in the NSBRI Team Workshop and Retreat of 1999 and 2000 that produced a needs assessment for the NSBRI Request for Applications (NSBRI 00-02): themes and specific research questions. Thus, although two of the team projects did not interact as closely as the other two, there was a genuine team effort in maintaining team integrity, spirit, and overall productivity.

2. Critical Evaluation of Level of Risk Reduction in Team Research Area

As a team, the investigators have made significant progress in performing biomedical research at a basic level that validates the early hypotheses sufficiently, so as to justify proceeding with the next 3-year cycle of NSBRI funding. In the case of the Shearer and Butel projects, these conjoint projects have demonstrated that it is extremely important to move on to another ground-based model of space flight to establish the potential risks of space radiation that might confront astronauts in long-term space travel. These new directions came about when it was discovered that a highly specific assessment of antibody function (response to bacteriophage

ϕX-174) was not altered in human subjects during the Antarctic winter-over, arguably the best ground-based model of space flight yet developed. Since this assay tests several aspects of T-cell function, as well as the ability of B-cells to produce antibodies, and because the completion of the assessment of the remainder of T-cell assays (lymphoproliferation to antigens, cytokine production by stimulated lymphocytes, lymphocyte subset distribution) on the remainder Antarctic blood specimens will take up to 1 year to complete, Dr. Shearer and Dr. Butel decided to develop a murine radiation and latent virus space model with NSBRI Radiation Team collaborators, Dr. Daila Gridley and Dr. Gregory Nelson. In an elegant series of peer-reviewed and published experiments, these two individuals have demonstrated that 3 Gy of either proton or gamma-radiation produced a severe reduction in both T- and B-cells in mice. In collaborative studies, we plan to infect irradiated mice with two latent murine viruses (gammaherpesvirus and polyomavirus) in order to assess the possibility that the latent virus would become activated due to the drop in immunosurveillance power secondary to radiation. Also with these collaborators at Loma Linda University, we plan to attempt development of a countermeasures program with chemical radiation blockers. These experiments will be initiated in the new cycle of NSBRI funding.

In the synergy project of the sleep-deprivation model of space flight, Dr. Shearer and Dr. Dinges clearly have shown that sleep-inducing cytokines and their receptors are elevated in human subjects totally deprived of sleep for 88 hours. Interestingly, subjects deprived of sleep for the same time interval, but permitted two 2-hour naps (12 PM - 2PM; 12 AM - 2 AM) did not show these changes. Thus, a possible risk reduction was suggested by prescription of short naps in the busy schedules of astronauts manning spaceships going to Mars or in astronauts taking a 1-year duty in the space schedule. Possibly, in a maneuver as simple as enforcing short nap times during the astronauts' busy schedules, the risk of elevation of blood cytokines could be prevented and the risk of reactivation of chronic (latent) viral infections and development of virus-driven malignancy could be reduced.

The planned interaction of Dr. Fox's project with Dr. Butel's might lead to the development of molecular probes that could detect microbial contamination of the spaceship with viruses. This project will require a renewed cycle of funding for both Drs. Butel and Fox, which appears to be a possibility. Preliminary discussions have already been held by these two project leaders for this purpose. Since Dr. Fox's project has an elevated Critical Research Path countermeasures readiness level of 5 (demonstration to prove efficacy), successful collaboration would ensure rapid assessment of its efficacy.

3. Programmatic Implications of Individual Project Results

The accomplishments of the individual research projects of the Immunology, Infection, and Hematology Team have clearly identified two and possibly three strong, interactive project leaders who have demonstrated an excellent ability to work as team members, rather than just as individual project members. Members of the "Space Flight Immunodeficiency" project (Shearer) and "Immune Function and Reactivation of Latent Viruses" project (Butel) have the common goal of trying to determine the Critical Research Path risks to space flight in regard to development of immunodeficiency, infections, and cancer. Dr. Fox's research deals with methodology of microbe (bacteria) detection, somewhat related to the goals of the Shearer and Butel projects. Dr. Alfrey's project has attempted to define the molecular mechanism of space "anemia" (other than the simple explanation of fluid shifts). In the context of the bone marrow

pleuripotent stem cell that can differentiate into all blood cells (lymphocytes, neutrophils, erythrocytes, etc.), this project is related to the others. The Immunology, Infection, and Hematology Program identified bone marrow stem cell biology as one of its eight themes for the recompetition of NSBRI funding. Thus, research directed at the impact of space flight conditions upon the total development of the bone marrow stem cell would fit very well into the programmatic theme of the team. In that sense, all of the initial projects of the program contributed to a unified concept. Clearly, the future projects of the program should more closely focus on the central theme of host defense in space travel: detection of alterations in host defense using ground-based space models, identification and quantitation of the types of microbial infections and cancer that take advantage of a compromised immune system, and identification of the earliest stage of stem cell development where space-induced damage occurs. Incumbent upon these discoveries will be the development of suitable countermeasures that will prevent these injuries from happening. Indeed, there are several powerful countermeasures currently available in clinical immunology, infectious diseases, and hematology/oncology that might lend themselves for this purpose. Replacement of antibody immunity by passive immunization (intravenous immunoglobulin), employment of microbe-specific pharmaceutical agents, and autologous stem cell transplantation could be adapted for space flight if the risks of host defense deficiency, chronic infections, and premature malignancies are shown to be unacceptably high.

William T. Shearer, M.D., Ph.D.
Program Team Leader

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE "IMMUNOLOGY, INFECTION, AND HEMATOLOGY" FINAL PROGRAM REPORT 2000

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I. PROGRAM RESEARCH ACCOMPLISHMENTS

A. National Recognition of Space Flight Immunology Research

The team members of the overall program have made substantial accomplishments in the past 3 years (2.5 years of support for the "Space Flight Immunodeficiency" project). Two important publications will be made of the synergy projects involving interactive collaboration with Dr. Desmond Lugg (ANARE) in Australia and Dr. David Dinges (University of Pennsylvania) in Philadelphia. These publications will appear in the January 2001 issue of the *Journal of Allergy and Clinical Immunology*, as part of a new direction for clinical immunology. Dr. Gerald Sonnenfeld, the most prolific researcher in immunology research of astronauts in space travel or in ground-based models, has been asked to write an editorial on the two publications, one dealing with neoantigen T-cell dependent antibody responses in humans in the Antarctic winter-over model of space flight, the other with the peripheral blood concentrations of soluble tumor necrosis factor-alpha receptor I and interleukin-6 in the sleep deprivation model of space flight (both factors induce sleep in animals and human study subjects, and both were elevated in humans deprived of sleep). Moreover, as a new feature of this journal, there will be direction given in both papers to alert the reader to the availability of a website video on the space flight conditions and ground-based model systems used to make the observations in the publications (NSBRI video edited by Dr. Shearer and Ms. Kathy Major, NSBRI Public Relations). This website video has been acclaimed by *Journal* editor Dr. Donald Y.M. Leung as one of the most innovative new features in scientific publication history and will serve as a model for enhancement of outstanding scientific publications in the future. Thus, the synergy projects of the Immunology, Infection, and Hematology Team have brought new recognition to the NSBRI over and above the rewards of publication. By featuring NSBRI-funded space immunology research in the first issue of the next millennium, the editor, Dr. Donald Y.M. Leung, and team leader Dr. Shearer, wish to draw the attention of clinical immunologists to the new and exciting area of space immunology research. Adaptation of the human immune response system to the challenges of long-term space travel, consequently, is an important research area for the next century that will record the exploits of humans in interplanetary space travel. Prediction of the immune risks of such space travel and the development of a suitable countermeasures program must assume a very high priority in the NSBRI overall program and demand the attention of the best scientists in the world. By making these publications and contributing to this feature presentation in the best clinical immunology journal in the world (15,542 citations in 1999), the Immunology, Infection, and Hematology Team has significantly raised the awareness of the scientific and clinical public to the challenges and opportunities in space immunology research.

B. International Collaboration in Virus Research

Another major accomplishment of the overall program has been the international collaborative research initiated by Dr. Janet Butel and supported by Dr. Shearer. Dr. Butel has obtained study subject specimens (blood, urine, saliva) from the Russian Institute for Biomedical Problems in Moscow to study the effects of 6 months of human encapsulation upon virus shedding, virus DNA quantitation, and cytokine production. Dr. Shearer participated in this last study. Thus, two of the program's individual projects collaborated with Russian space immunology researchers in an ongoing study. This international collaborative research of the

program members documents the ability of the overall program to go beyond its original goals and objectives and obtain critical new important data that would not be possible if the team projects operated in just an isolated style. This NSBRI-Russian venture will lead to additional opportunities for research, many of which future NSBRI program investigators may enjoy.

C. Summary

The program of the NSBRI Immunology, Infection, and Hematology Team has demonstrated the ability and commitment of individual project investigators to work synergistically to produce data that stand-alone projects would never achieve. The efforts of the four project principal investigators have produced a needs assessment that has identified a critical need for the extension of this program's efforts in the future of the NSBRI. These early successes predict a good future for the investigators selected by peer-review to take the program into the next three years.

II. RISK REDUCTION ACHIEVED BY PROGRAM

The risk reductions in the Critical Research Path have been the identification to use additional ground-based space models to test conditions of space flight not examined with the present models. Selection of the irradiated and virus-infected mouse model will enable program members Shearer, Butel, and possibly new members to assess the risks of solar radiation on space travelers. By collaborating with members of the Radiation Program, it will be possible to leap ahead and gain access to new information that may radically lower the risk of astronauts to the 2 Gy proton and gamma radiation that they are likely to receive on the voyage to Mars.

The synergy project of the Immunology, Infection, and Radiation Team (Shearer, Butel) and Human Performance, Chronobiology, and Sleep Team (Dinges, Mullington) has identified a possible countermeasures program (short intermittent naps) to prevent the possibly harmful effects of elevation of the high affinity tumor necrosis factor-alpha and interleukin-6 in the blood of sleep-deprived subjects. Additional experiments planned for the future will determine whether this is a valid countermeasure capable of lowering the Critical Research Path risks of immunodeficiency, infection, and cancer.

The work of Drs. Fox and Alfrey has contributed additional important information to the eventual lowering of the risks of bacterial contamination of the spaceship and the anemia of space flight.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
FINAL PROJECT REPORT 2000

PROJECT TITLE: Space Flight Immunodeficiency

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William T. Shearer, M.D., Ph.D.

William T. Shearer, M.D., Ph.D.
Project Principal Investigator

EXECUTIVE SUMMARY

1. Key Findings and Discoveries

1.1 Antarctic Winter-Over Model of Space Flight

In collaboration with Dr. Desmond J. Lugg, Head of Polar Medicine, Australian Antarctic Division, Australian National Antarctic Research Expedition (ANARE), we have performed the first assessment of human specific antibody response to a T-cell dependent neoantigen (i.e., bacteriophage ϕX-174) in a space flight model. Previous investigations of humans in space flight or in models of space flight measured only serum immunoglobulin concentrations that do not equate to antibody. The bacteriophage ϕX-174 vaccine is known as the gold standard for evaluating deficiencies of humoral (antibody) immune responses. Therefore, this study was particularly important in quantitating several aspects of human immune responses (e.g., viral clearance, primary [IgM] antibody, secondary [IgG] antibody, and helper T-cell-induced immunologic switching of IgM to IgG production). The conditions of the Antarctic winter-over include stress, isolation, containment, and microbial contamination, which are common to those of space flight but lack the conditions of microgravity and solar radiation. Nevertheless, the Antarctic model is one of the best ground-based models of space flight. With the collaboration of ANARE, we studied test subjects stationed at the Casey outpost in Antarctica and control subjects stationed on Macquarie Island. Macquarie Island subjects are considered suitable controls because they have access to the mainland during the winter, in contrast to those subjects at Casey. In the third month of the Antarctic winter-over, subjects were immunized with bacteriophage ϕX-174 and 6 weeks later were given a booster injection. At Casey, the test subjects' immune responses were equivalent to those of the control subjects on Macquarie Island and to the absolute control data in the testing laboratory of Dr. Hans Ochs at the University of Washington, Seattle. These humoral immune responses do not show the deficiencies of some cellular immune responses (delayed type hypersensitivity [DTH] skin test responses to recall antigens) observed by Dr. Lugg in previous ANARE expeditions. These data are important because they clearly define normal antibody responses to a neoantigen (bacteriophage ϕX-174) during the rigorous conditions of the Antarctic winter-over. Also, they clearly do NOT predict similar normal humoral immune responses in space flight because of the important lack of the space factors of microgravity and solar radiation (protons and gamma-rays). These results dictate the application of new models for the assessment of risks of space flight for producing antibody deficiencies. The animal model of irradiated mice challenged with vaccines or virus challenge would be one such example that will be explored in the next funding extension of the present grant. The Antarctic winter-over experiments will be featured in the January 2001 issue of the well-respected *Journal of Allergy and Clinical Immunology* that will introduce space flight immunology research to its readership, along with a cover illustration of space exploration and a National Space Biomedical Research Institute (NSBRI) videotape of the conditions of space flight and model systems utilized in the publication.

1.2 Sleep Deprivation Model of Space Flight

With the recommendation of the NSBRI External Advisory Council, we began a synergy project collaboration with another NSBRI Team (Human Performance, Chronobiology and Sleep) and Dr. David F. Dinges, Professor of Experimental Psychiatry, University of

Pennsylvania. Dr. Dinges had previously demonstrated alterations in certain immune cells and cytokines in human subjects deprived of sleep for 64 hours. The loss of sleep in space flight is a major problem for astronauts, as judged by the fact that the most commonly prescribed medication is sleeping pills. In the synergy project, we measured plasma cytokines in a cohort of human subjects with total sleep deprivation (TSD) or partial sleep deprivation (PSD) for 88 hours. Two sleep regulatory cytokines/cytokine receptors (soluble tumor necrosis factor-alpha receptor 1 [sTNF-αRI] and interleukin 6 [IL-6]) were shown to become elevated in the TSD subjects compared to the PSD subjects. The same two messenger molecules of the neuroendocrine-immune system have been shown to regulate sleep in small animal studies involving instillation in the cerebral cortex. The fact that the PSD subjects did not demonstrate an increase in sTNF-αRI and IL-6, suggests that the short naps interspersed throughout the sleep deprivation period might serve as the basis for a countermeasures approach to the problems of loss of cognitive and mechanical ability in sleep-deprived humans. These exciting results have lead to plans for new synergy projects with Dr. Dinges (now Co-Leader of the NSBRI Neurobiology and Psychosocial Factors Team), involving assessment of upregulation of target cell virus receptors by alterations in peripheral blood cytokines and chemokines. The results of the first study will be co-featured with those of the Antarctica study in the January 2001 issue of the *Journal of Allergy and Clinical Immunology.*

1.3 Anti-Orthostatic Suspension Murine Model of Space Flight

Dr. Wayne Smith and colleagues have made several important observations on acquired and innate immune responses using the anti-orthostatic suppression (AOS) mouse system, which mimics known effects of space flight (i.e., underuse of lower extremities, overuse of upper extremities, limited excursion, containment, and cephalad (head) fluid shift. Dr. Smith was able to show that the AOS mouse produced greater DTH skin test responses to a recall antigen than control mice (orthostatic suppression or untethered). Although these findings may be somewhat confounded by the cephalad fluid shift, they suggest that AOS suppression may accentuate hypersensitivity reactions as part of an induced imbalance in immune homeostasis. It is possible that these data indicate a need to assess by additional model studies the development of autoimmune disease in astronauts in long-term space flight.

In addition, Dr. Smith has demonstrated that endotoxin challenge of AOS mice induced a greater expression of the intracellular adhesion molecule type-1 (ICAM-1) in muscle tissue deprived of fluid. This situation is likely to lead to an influx of leukocytes by virtue of their attraction by ICAM-1 molecules on endothelial cell layers. The muscle atrophy observed in astronauts might be related in part to a possible reperfusion injury due to leukocyte infiltration of blood-deprived muscle tissue.

Finally, as a follow-on study of the conflicting studies in the space research literature where phagocytosis and microbicidal activity of neutrophils were examined in the AOS mouse, Dr. Smith was unable to demonstrate any clear effects of AOS upon oxidative function of peritoneal neutrophils. This study will be published in *Aviation, Space, and Environmental Medicine.*

2. Satisfaction of Hypotheses, Objectives, Specific Aims of Original Proposal

Specific Aim 1 had two objectives: 1) Evaluate ANARE subjects for evidence of immunodeficiency, and 2) Evaluate Johnson Space Center (JSC) capsule-isolated astronauts-in-training for evidence of immunodeficiency. Only the first objective was carried out, because the JSC capsule studies were postponed by the National Aeronautics and Space Administration (NASA). Of the studies proposed on ANARE subjects, we have completed the assessment of specific antibody function, but we are now assessing cellular immune responses: lymphocyte subsets, mitogen and antigen-induced lymphoproliferation, and cytokine production. The 1999 ANARE specimens of frozen plasma and cells reached us in January - March of 2000 (3 separate shipments), so most of the cellular studies are in progress. Also, critical to the evaluation of cellular immunity is the viability of the cell specimens. Regardless of viability, it will be possible to measure cytokine expression by DNA/RNA technology in the cells

Specific Aim 1 was modified by inclusion of sleep deprivation experiments in the synergy collaboration project described above. Both the Antarctic and sleep-deprivation studies were sustained by the same hypothesis, namely that conditions of space flight models on Earth would induce alterations in the human immune system that might indicate the need to anticipate defects of immunity developing in astronauts on long space voyages (e.g., 3-year trip to Mars). In the first objective, the Antarctic antibody studies did not validate the hypothesis, but they pointed to the need to change the model so that space factors not present in the Antarctic winter-over model could be examined. Thus, in the new grant cycle, we will use irradiated mice challenged by latent virus (gammaherpesvirus and polyomavirus), in collaboration with Dr. Daila Gridley and Dr. Gregory Nelson, radiation biologists at Loma Linda University. The modified objective of the first specific aim (sleep- deprivation studies) did validate the hypothesis, because it showed that sleep deprivation of 88 hours induced significant alterations in cytokine messenger molecules that connect the neuroendocrine-immune system. These sleep deprivation-induced changes in TNF-αRI and IL-6 led to the development of the next hypothesis: altered chemokine levels in astronauts or in ground models of space flight will upregulate viral receptors on target cells and lead to chronic infection and possibly development of malignant clones of transformed lymphocytes, such as Epstein-Barr virus (EBV)-driven lymphomas. This new hypothesis will be tested in ongoing synergy projects, in collaboration with Dr. David Dinges.

Specific Aim 2 of the original project had several components, but the basic objective was to determine whether biochemical or structural components of the inflammatory system and cellular molecule adhesion system are altered in the AOS murine model of space flight. The underlying hypothesis was that local tissue fluid shifts associated with the AOS model would affect the tissue distribution of adhesion molecules and, therefore, alter inflammatory responses. This hypothesis was partially validated in the experiments to date, in that an enhanced DTH skin test response to a recall antigen was seen in the AOS mouse, and an upregulation of endotoxin-induced ICAM-1 molecules was documented in fluid-deprived muscle tissue. These results indicate a need to pursue the important research area of muscle reperfusion, since it is well known that astronauts suffer from muscle atrophy.

3. Implications of Project Research for Risk Reduction in Critical Research Path

The Critical Research Path Risks addressed by **Specific Aims 1 and 2** in this research project were: 1) immunodeficiencies/infections (rank 1 [high priority], type III [problem

suspected]) and 2) carcinogenesis (rank 1, type III). Thus, we intended to demonstrate whether there should be concern for the possible harmful effects of known conditions of long-term space flight. Our progress to date leads us to believe that continuation of this plan is very important for the preparation for interplanetary space travel. The unknown effects of microgravity and solar radiation (estimated 2 Gy) upon the normal balance of immunity needs careful exploration with additional models of space flight. There is ample clinical evidence from patients on Earth that the immunosurveillance system, when suppressed by virus infection (e.g., AIDS), by therapeutics (e.g., corticosteroids, immunomodulators, cytotoxic agents), by radiation exposure, and by unremittant stress (e.g., family caregivers to patients with terminal diseases such as Alzheimers) begins to fail in protecting the host against opportunistic infections (e.g., Pneumocystis carinii pneumonia), reactivation of latent viral infections (e.g., EBV infection), and malignancies (e.g., lymphomas, leukemias). The purpose of pursuing these potential developments in astronauts subjected to the potential dangers of space flight is to first examine their feasibility, and to then devise a countermeasures program to negate their impact on astronauts. Currently, our countermeasures readiness level is 1 or 2 (basic research level), but our sleep-deprivation experiments have already suggested that short intermittent naps (2-hour naps every 12 hours) prevent the increased production of the neuroendocrine-immune system messengers involved in sleep regulation. This, at least in a preliminary sense, is a significant beginning to the development of a countermeasure for the immune consequences (infections, cancer) of sleep-deprivation in the long-term perspective. These findings also hold importance for a countermeasures program for diseases and conditions with immediate consequences on Earth (e.g., sleep-starved workers, truck drivers, and pilots who sustain accidents). The experiments with the AOS mouse model that demonstrated an upregulation of ICAM-1 molecules in fluid-deprived muscle tissue possibly may related to the muscle atrophy observed in astronauts. It is well known that in reperfused cardiac tissue after heart attacks, there is an upregulation of ICAM-1 molecules that leads to destructive infiltration of leukocytes. Possibly, the same mechanism operates in space-flight muscle atrophy as part of the muscle disuse mechanism.

We are delighted to be able to anticipate the continuation of these important experiments in the next 3 years of NSBRI grant support.

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Project Principal Investigator

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
"SPACE FLIGHT IMMUNODEFICIENCY"
FINAL PROJECT REPORT 2000

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I. PROJECT RESEARCH ACTIVITY

A. SPECIFIC AIM 1

Specific Aim 1: Determine whether a new type of secondary immunodeficiency (Space Flight Immunodeficiency) is likely to develop in astronauts exposed to long-term space travel using ground-based analogs to space flight.

1. Hypothesis

Conditions of long-term space travel (stress, isolation, containment, sleep deprivation, microgravity, microbial contamination, solar radiation) will weaken the immune system so that it can no longer defend the host against infection and malignancy.

2. Objectives

2.1 Examine immune responses in subjects exposed to the Antarctic winter-over model of space flight.

2.2 Examine immune responses in pre-astronauts contained for 6-months in a NASA/JSC capsule (subsequently postponed by NASA).

2.3 Use the synergy project approach to determine alterations in plasma cytokines and cytokine receptors in the sleep deprivation model of space flight.

3. Antarctic Winter-Over Model of Space Flight

The present grant ("Space Flight Immunodeficiency") was funded in April 1998. Travel and transport of reagents and supplies for these experiments started in November 1998 (end of Antarctic winter, beginning of Antarctic summer; travel to Australian Antarctic outposts not possible during winter) and continued through February 1999. Experiments were conducted during the Antarctic winter of 1999, and frozen (-70°C) plasma and frozen (-80°C liquid nitrogen) preserved peripheral blood mononuclear cell (PBMC) specimens were returned on dry ice to ANARE headquarters in Tasmania (December 1999 – February 2000) and then shipped on dry ice by air express to the Johnson Space Center in Houston, and from there to Baylor College of Medicine (BCM) in Houston (Virology – Dr. Janet S. Butel; Immunology – Dr. Howard M. Rosenblatt). Frozen plasma specimens (pre- and post-immunization with bacteriophage ϕX-174) were shipped by air express in May 2000 to collaborator Dr. Hans D. Ochs in Seattle, where this analysis is ongoing. The rest of the >1,500 samples are being prepared for analysis by Dr. Rosenblatt and Dr. James M. Reuben at the M.D. Anderson Cancer Center (1 block from BCM).

In addition to experimental subjects stationed at the 3 ANARE Antarctic outposts (Mawson, Davis, Casey), control subjects stationed on the supply island (Macquarie Island, 2000 miles from the Antarctic continent), provided plasma and PBMC specimens. Dr. Desmond Lugg and his collaborators had previously determined that the Macquarie Island subjects, who experience the harsh winter climate but who have access to Australia, had normal DTH skin test responses to recall antigens in contrast to the Antarctic outpost-bound experimental subjects. The immunological assays to be performed on the 1999 ANARE samples are listed in **Table 1**.

Dr. Janet S. Butel, Principal Investigator of the current Immunology and Infection Team project, "Reactivation of Latent Viruses", has also participated in the 1999 ANARE investigation. She and her colleagues are analyzing plasma, white blood cells (WBCs), saliva, and urine for evidence of reactivation of latent viruses such as EBV, polyomavirus, and BK virus. We are collaborating with the virology investigators at BCM to assess immune function in those Antarctic subjects showing evidence (shedding of virus, increased antibody titers, increased viral DNA content of WBCs). Thus, in patients where there are common specimens, we will be able to measure specific T-cell lymphoproliferative responses to latent viral antigens, e.g, EBV. Should there be immune suppression in patients with activation of latent virus infection, the cause-and-effect relationship of depressed immunity and reactivation of latent infections will be strengthened. We estimate that it will take up to 1 year to complete the analyses of Antarctic specimens, at which time it might be possible to devise more precise experiments for the next available expedition to the Antarctic, most likely ANARE 2002.

Table 1
PROPOSED STUDIES OF HUMAN SUBJECTS
ON THE AUSTRALIAN NATIONAL ANTARCTIC RESEARCH EXPEDITION*

1. Specific Antibody Formation

ϕX-174 bacteriophage (neo-antigen)

- primary immunization: month 5.0
- secondary immunization: month 6.5
- periodic blood samples: months 5, 5.25, 5.50, 6.5, 6.75, 7.0
- storage of serum at -70°C
- batch analysis of IgM, IgG responses

2. ** **Specific T-Lymphocyte Responses**

a. Specific Antigens
- Candida
- Streptokinase-Streptodornase
- Tetanus

b. Non-Specific Mitogens (proliferation controls)
- PHA
- Concanavalin A
- Poke Weed Mitogen

c. Viral specific responses
- EBV, CMV, HSV proliferation

d. Time Schedule
- Periodic blood samples: months 0, 2, 4, 6, 8

3. *** **Extended Flow Phenotyping (same time schedule as 2)**

a. Naïve Lymphocytes
- CD4 CD45RA
- CD8 CD45RA

b. Memory Lymphocytes
- CD4 CD45RO
- CD8 CD45RO

c. Cytotoxic T-Lymphocytes
- CD8 CD28

d. Activated T-Lymphocytes
- CD8 CD38

4. ** **Cytokine Secretion (mRNA, Protein) (same time schedule as 2)**

a. TH1
- IL-2
- IFN-γ

b. TH2
- IL-4
- IL-10
- IL-6
- TNF-α

* Controls are project subjects studied identically but living on Macquarie Island. Dr. Rosenblatt will perform Tests 1, 2, and 3; Dr. Reuben will perform Test 4.

** These tests will be performed on blood specimen cells preserved in dimethyl sulfoxide solution (DMSO) and frozen in liquid N_2 at -80°C.

*** Flow cytometry studies will be performed on PBMCs preserved in DMSO-preserved and liquid N_2 frozen at -80°C.

We are particularly concerned about the viability of the frozen/preserved cells, since >50% viability will be essential to studying surface markers, lymphoproliferation, and stimulated-cell cytokine production. If cell viability falls below this threshold, we plan to measure the RNA for constitutive mRNA message expression. Since this would require less than 1 year to accomplish, we will move on to the irradiation mouse model component of the new grant period.

The first batch of data derived from the 1999 ANARE studies in the Antarctic is now presented. Eleven adult subjects, ages 30 to 57 years, were given 2 intravenous immunizations with bacteriophage ϕX-174 (0.02 ml/kg of a standard preparation, 1 X 10^{11} phage particles/mL). Five months into the winter-over period, the first immunization was given (7/13/99), and 6 weeks later the second immunization was given (8/24/99). Post-immunization blood draws were taken **(Table 2)**. There were no side-effects experienced by any of the subjects receiving immunization.

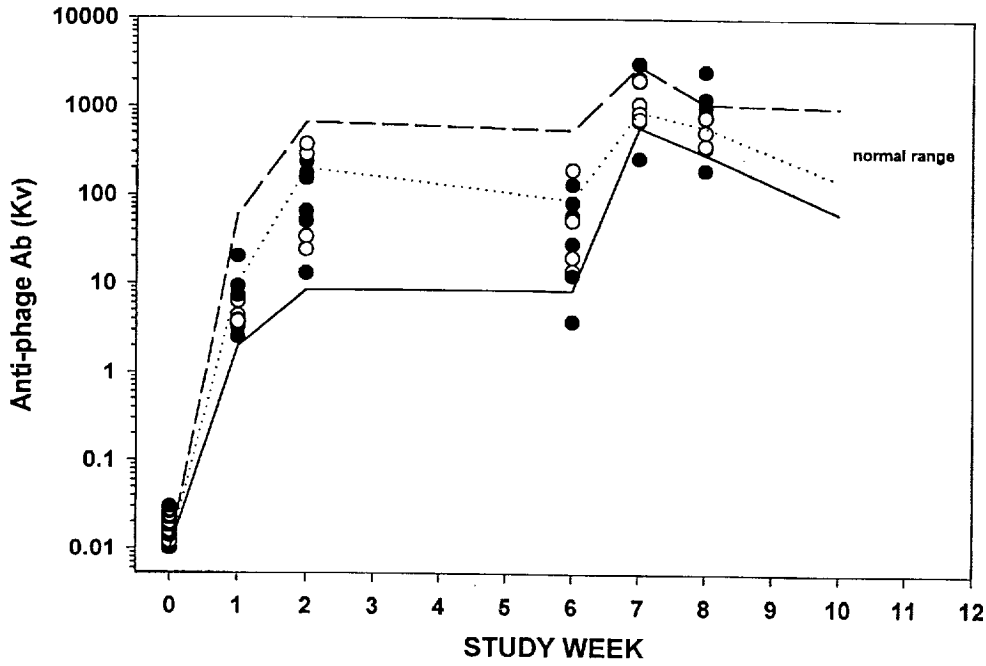
Table 2. ANARE/NSBRI Research Volunteers, Casey, Antarctica 1999

				Bacteriophage ϕX-174 Immunization Dates / Samples Numbers						
				Primary Immunization				Secondary Immunization		
Subject No.	Age (yrs)	Height (cm)	Weight (kg)	1 Pre (mo/day)	2 Post 15 Mins	3 Post 1 Wk	4 Post 2 Wks	5 Pre	6 Post 1 Wk	7 Post 2 Wks
1	57	180.	103.6	7/13	7/13	7/20	7/27	8/24	8/31	9/7
2	50	189.5	87.3	7/13	7/13	7/20	7/27	8/24	8/31	9/7
3	33	177.5	104.9	7/13	7/13	7/20	7/27	8/25	8/31	9/7
4	31	189.5	87.7	7/13	7/13	7/20	7/27	8/24	8/31	9/7
5	56	185	95.9	7/13	7/13	7/20	7/27	8/24	8/31	9/7
6	38	182	108.0	7/13	7/13	7/20	7/27	8/24	8/31	9/7
7	50	172	87.5	7/13	7/13	7/20	7/27	8/24	8/31	9/7
8	43	191.5	124.0	7/13	7/13	7/20	7/27	8/24	8/31	9/7
9	30	175	80.0	7/13	7/13	7/20	7/27	8/25	8/31	9/7
10	32	181	78.8	7/13	7/13	7/20	7/27	8/25	8/31	9/7
11	43	173.5	82.0	7/13	7/13	7/20	7/27	8/25	8/31	9/7

The plasma samples were analyzed for bacteriophage ϕX-174 clearance, primary and secondary antibody response to bacteriophage ϕX-174, memory amplification, immunoglobulin class of the secondary antibody response, and the response classification **(Figure 1)**. All of the subjects were able to normally clear the bacteriophage by 1 week after immunization, all made normal primary antibody responses, and all subjects made normal secondary antibody responses with memory amplification and switch from IgM to IgG antibody production, with 1 subject showing a high normal and 2 subjects a slightly low normal pattern. The response classification was 8 subjects: normal, 1 subject atypical (due to high antibody response), and 2 subjects probably normal (slightly low antibody response). The Australian control subjects' plasma yielded the same results. Thus, the data do not support the hypothesis that *de novo* specific antibody responses of subjects become defective during the conditions of the Antarctic winter-over. The bacteriophage ϕX-174 study tells us

not only about specific antibody function but also about helper T-cell function since the switch from IgM to IgG production is mediated in part by helper ($CD4^+$) T-cells. These findings do not rule out the possibility of defects in other components of immunity, such as T-cell response to antigens.

Figure 1
ANTIBODY RESPONSES (CASEY)



Subjects began the ANARE winter-over at the Antarctic Casey Outpost on March 1, 1999. The 1st immunization with the bacteriophage X-174 was given on July 13, 1999 (study week 0); the 2nd immunization was given on August 24, 1999 (study week 6). Blood samples for antibody assays were obtained at the times indicated prior to and after each immunization. Each symbol represents the total antibody titer for an individual at the indicated time point. The normal range represents maximum (dashes), median (dots), and minimum (solid lines) values, respectively, for normal subjects in Dr. Ochs' clinic and laboratory.

4. 1998-2000 Experiments: Alteration of Plasma Cytokines/Cytokine Receptors in Sleep-Disrupted/Deprived Humans

In response to the NSBRI External Advisory Council's earlier (1998) recommendation that short-term experiments should be undertaken by the investigators of the "Space Flight Immunodeficiency" project, in light of the NASA postponement of our January 1999 Capsule Study (6-month mock isolation of space), and the long-term nature of Antarctic studies, a synergy collaboration was made with the NSBRI Human Performance Factors, Sleep, and Chronobiology Team (Dr. David F. Dinges – University of Pennsylvania, Dr. Janet M. Mullington – Harvard Medical School).

Convincing arguments have been made to support the hypothesis that the inflammatory cytokines IL-1β, IL-6, and TNF-α are involved in sleep-awake regulation, and that their administration produces sleepiness and fatigue. This evidence constitutes the basis for pursuing these sleep disruption/deprivation and cytokine alteration studies. TNF-α and

IL-1β are known to mediate non-rapid eye movement sleep through activation of NFkB, a DNA-binding protein involved in transcription. Awake cycles and sleep-disruption (or deprivation) produce high plasma levels of IL-1β and IL-6, as well as activation of NFkB. High plasma levels of TNF-α upregulate the T-cell/monocyte surface expression of TNF-αRI and TNF-αRII, as well as the release of these receptors in soluble form as part of a homeostatic mechanism. After NFkB activation, other cytokines such as IL-4, IL-10, and IL-2 are known to sequentially play a role in sleep-awake cycles. IL-2R is upregulated on T-cells and is shed in its soluble form as part of the overall regulation process.

As part of a sleep-disruption/deprivation study supported by the United States Air Force, volunteers were either prevented from sleeping for 88 hours (TSD) or sleep-disturbed by permitting 2-hour naps every 12 hours over 88 hours (PSD). Blood was sampled throughout the study period and the plasma stored at -70°C for the present study; plasma specimens obtained at 6-hour intervals were analyzed. The number of study subjects for each condition and cytokine/cytokine receptor measured is given in **Table 3**. TSD and PSD numbers represent the pooled total number of subjects taking low-dose caffeine (0.3 mg/kg/hour) and those taking a placebo. Pooling the treatment group was justified by the absence of any significant differences between caffeine and placebo groups, as illustrated by the plasma levels of TNF-αRI in the comparison of the TSD subjects vs. the TSD + caffeine subjects **(Figure 2)**.

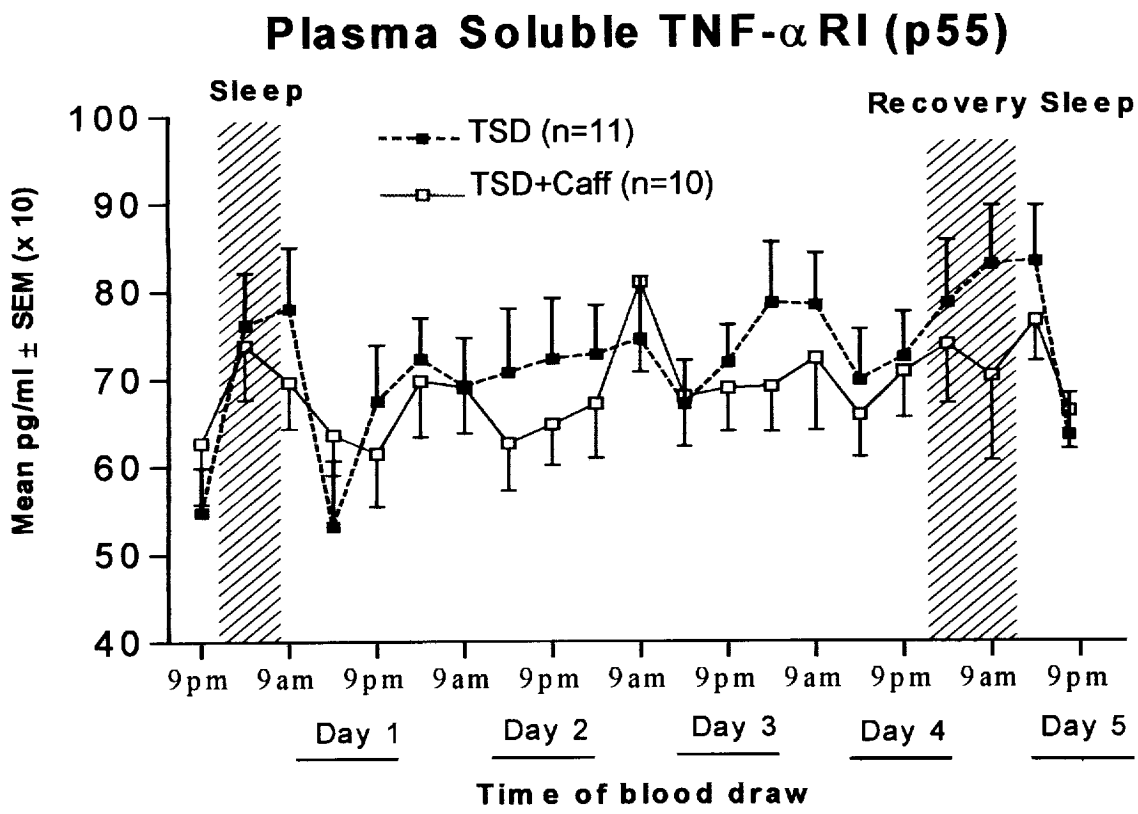
Table 3
Number of Subjects Per Study Group and Measurements in 88-Hr Sleep Disruption/Deprivation Study *

Groups	TNFα-RI	TNFα-RII	IL-6	IL-2R	IL-10	TNFα
TSD	21	21	19	10	10	9
PSD	21	21	21	13	13	14

* *All subjects went through an identical normal 24-hour period of sleep and awakeness (baseline evaluation) before being separated into the 2 study groups, TSD and PSD.*

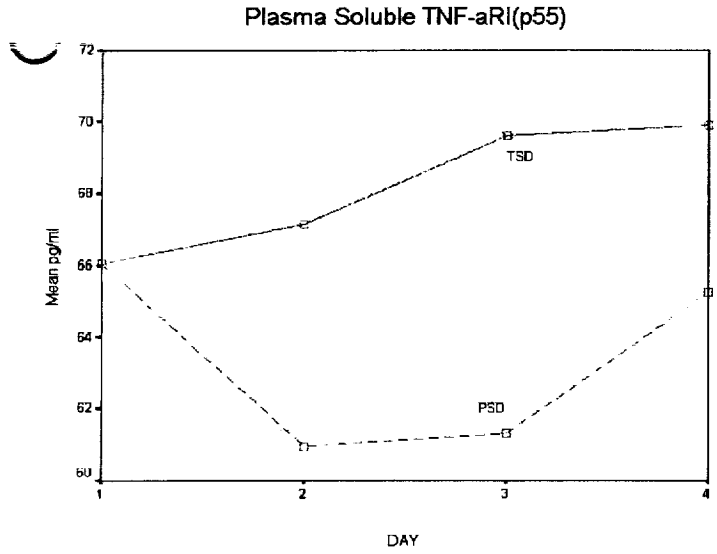
Figure 2 demonstrates an additional elevation of TNF-αRI during sleep which falls to a nadir during daytime. This normal rhythmicity is lost in sleep deprivation but regained after recovery sleep. ELISA assays were used to measure the cytokine/cytokine receptor concentration in the plasma. Analysis of variance and covariance for repeated measures with polynomial trend analysis was used to assess the effects of time (4 days), TSD vs. PSD, and interactions among these on immune measures. Day 1 measures were treated as covariates in the analysis so that all groups started at the same mean value. All of the 4 values for individual times of each day were calculated as a single value to reduce interassay variability. Polynomials were, in effect, fitted separately to each subject's measures across time in order to assess time trends within individuals and the effects of sleep deprivation. A log transformation was applied to IL-6 data prior to analysis, due to lack of normality. Log transform did not affect the results of the other measures.

Figure 2



An interaction between the effects of time and sleep deprivation level (P=0.01) was detected for TNF-αRI (p55) **(Figure 3)**. Although small, there was a significant increase over time in the TSD group, but not the PSD group. There were no statistically significant effects of time or sleep disruption/deprivation for the plasma soluble TNF-αRII (p75) over the 4-day period **(Figure 4)**. An interaction between the effects of time and sleep deprivation level (P=0.03) was detected for plasma IL-6; there was a linear increase over time in the TSD group, with no increase in the PSD group **(Figure 5)**. An overall linear time increase in plasma soluble IL-2R was suggested (P=0.07) (data not shown). This increase was not associated with the level of sleep deprivation. There were no statistically significant effects of time or sleep deprivation for plasma IL-10 and TNF-α, although the variance of interassay measurements presented confirmation of an increased trend in TSD over the 4-day period (data not shown). Although not reported in this application, all subjects were given comprehensive, psychological, and cognitive/motor coordination tests throughout the period of accumulating sleep deprivation. These data demonstrate that the stress of 88 hours of sleep disruption/ deprivation in healthy volunteers produced significant increases in TNF-αRI and IL-6, cytokines that regulate sleep and mediate messenger systems between the neurological, endocrine, and immune systems. In particular, TNF-αRI increased in response to TSD as contrasted to PSD. Moreover, there was a time-dependent increase in the plasma level of soluble TNF-αRI as contrasted to its baseline concentration.

Figure 3

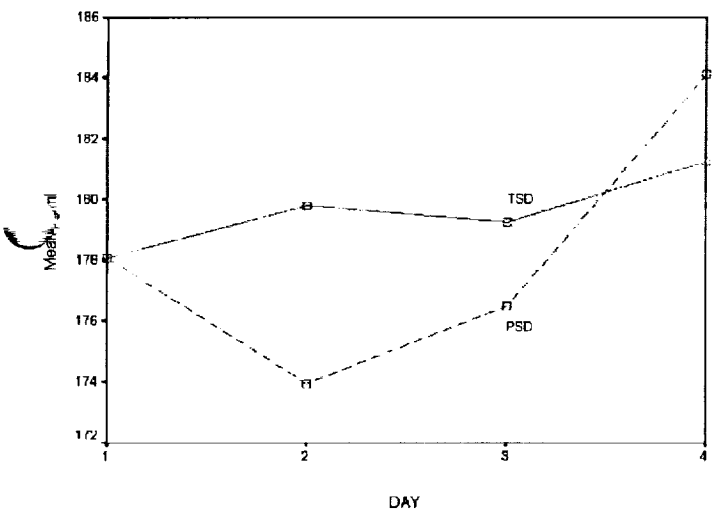


Measure: P55

T1P0P55	Day	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
.00	1	66.054[a]	.000	66.054	66.054
	2	60.945[a]	2.079	56.732	65.158
	3	61.301[a]	2.083	57.080	65.521
	4	65.254[a]	2.121	60.956	69.552
1.00	1	66.054[a]	.000	66.054	66.054
	2	67.156[a]	2.103	62.894	71.417
	3	69.618[a]	2.107	65.349	73.888
	4	69.900[a]	2.146	65.553	74.248

a. Evaluated at covariates appeared in the model: BASEP55 = 66.054.

Figure 4

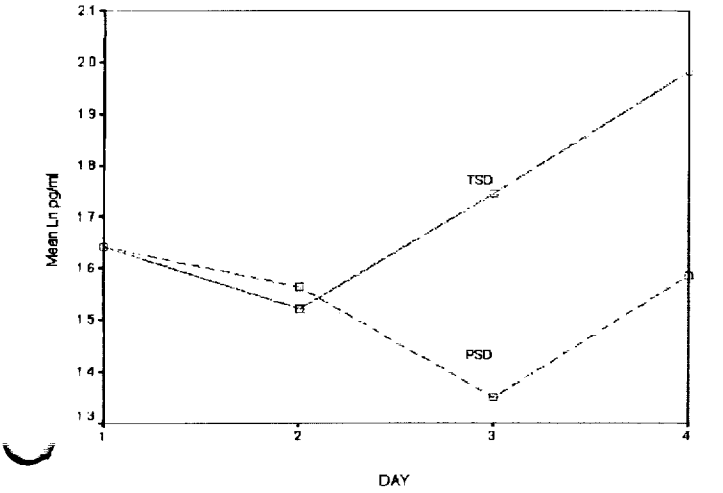


Measure: P75

TSD1PSD0	Day	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
.00	1	178.100[a]	.000	178.100	178.100
	2	173.948[a]	3.855	166.138	181.758
	3	176.487[a]	5.780	164.775	188.200
	4	184.113[a]	6.600	170.740	197.485
1.00	1	178.100[a]	.000	178.100	178.100
	2	179.803[a]	3.943	171.815	187.791
	3	179.243[a]	5.912	167.264	191.223
	4	181.269[a]	6.751	167.591	194.947

a. Evaluated at covariates appeared in the model: BASELINE = 178.1001.

Figure 5



Measure: LNIL6

TSD1PSD0	Day	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
.00	1	1.643[a]	.000	1.643	1.643
	2	1.564[a]	.131	1.299	1.829
	3	1.350[a]	.165	1.015	1.685
	4	1.585[a]	.147	1.288	1.882
1.00	1	1.643[a]	.000	1.643	1.643
	2	1.521[a]	.132	1.253	1.788
	3	1.746[a]	.167	1.409	2.084
	4	1.984[a]	.148	1.684	2.283

a. Evaluated at covariates appeared in the model: BASELINE = 1.6428.

5. Relation of Research Findings to Specific Aims, Hypotheses, and Objectives

Specific Aim 1 had two objectives: 1) Evaluate ANARE subjects for evidence of immunodeficiency, and 2) Evaluate JSC capsule-isolated astronauts-in-training for evidence of immunodeficiency. The second objective was not carried out, because the JSC capsule studies were postponed by NASA. We have completed the assessment of specific antibody function in the first objective. The first assessment of cellular immune responses: lymphocyte subsets, mitogen and antigen-induced lymphoproliferation, and cytokine production has been delayed because the 1999 ANARE specimens of frozen cells reached us in January - March of 2000 (3 shipments), so most of the cellular studies are in progress. We first need to evaluate the viability of the cell specimens, since this is a critical issue for functional studies. Regardless of viability, it will be possible to measure cytokine expression by DNA/RNA technology.

Specific Aim 1 was modified by inclusion of sleep deprivation experiments in the synergy collaboration project described above. The sleep-deprivation studies were sustained by the same hypothesis as the Antarctica studies, namely that conditions of space flight models on Earth would induce alterations in the human immune system that might indicate the need to anticipate defects of immunity developing in astronauts on long space voyages (e.g., 3-year trip to Mars). The Antarctic antibody studies did not validate the hypothesis but suggested a need to change the model, so that space factors (e.g., radiation) not present in the Antarctic winter-over model could be evaluated. We will use irradiated mice challenged by latent virus (gammaherpesvirus and polyomavirus), in collaboration with Dr. Daila Gridley and Dr. Gregory Nelson, radiation biologists at Loma Linda University. The sleep-deprivation studies validated the hypothesis, because it showed that sleep deprivation of 88 hours induced significant alterations in cytokine messenger molecules that connect the neuroendocrine-immune system. Our findings of changes in TNF-αRI and IL-6 in sleep deprivation have led us to develop the next hypothesis: altered cytokine levels in astronauts or in ground models of space flight will upregulate viral receptors on target cells and lead to chronic infection and development of malignant clones of transformed lymphocytes, such as EBV-driven lymphomas. This new hypothesis will be tested in ongoing synergy projects, in collaboration with Dr. David Dinges.

B. SPECIFIC AIM 2

Specific Aim 2: Determine whether biochemical or structural components of the inflammatory system and cellular adhesion molecule system are altered in an anti-orthostatic rodent model.

1. Hypothesis

Our hypothesis is that local tissue fluid shifts associated with anti-orthostatic suspension will effect the tissue distribution of adhesion molecules, and thereby alter the inflammatory response.

2. Objective

To determine whether biochemical or structural components of the inflammatory system and cellular adhesion molecule system are altered in an anti-orthostatic rodent model.

3. Specific Sub-Aims

3.1 To study the effect of anti-orthostatic suspension on parameters of baseline leukocyte recruitment.

Leukocyte recruitment is a key feature of inflammation. It is characterized by a distinct series of interactions between circulating leukocytes and vascular endothelial cells. Our first aim will be to determine if conditions of simulated space flight alter the kinetics of baseline leukocyte recruitment. We will use intravital microscopy to measure baseline leukocyte rolling, rolling velocity, adhesion and emigration in post-capillary venules of anti-orthostatically suspended (experimental), orthostatic and normal control mice. To date a detailed *in vivo* examination of leukocyte recruitment following anti-orthostatic suspension has not been reported.

3.2 To determine if leukocyte recruitment in response to a non-specific, acute inflammatory stimulus is altered by anti-orthostatic suspension.

Ischemia/reperfusion (I/R)in the cremaster muscle will be used as a model of non-specific inflammation. This model is well established and has been previously used by us and others to examine leukocyte-endothelial cell interactions in the cremasteric microcirculation. I/R-induced leukocyte recruitment will be assessed by intravital microscopy in anti-orthostatically suspended and control mice. The data generated will contribute to the understanding of the potential influences of space flight on generalized, non-specific inflammation.

3.3 To determine if anti-orthostatic suspension alters leukocyte recruitment during early and relatively delayed, antigen-specific inflammation.

A late phase allergic reaction (Type I hypersensitivity) will be induced in anti-orthostatic and control mice, by systemic sensitization and local challenge with chicken ovalbumin. In another series of experiments, a DTH reaction will be induced by topical sensitization and local skin challenge with dinitrofluorobenzene.

3.4 To determine if surface expression of endothelial cell and leukocyte adhesion molecules is altered by anti-orthostatic suspension.

Leukocyte recruitment depends almost entirely upon sequential and regulated interactions between endothelial cells and leukocytes via the expression of adhesion molecules. Therefore, we feel it is important to investigate the potential influences of simulated space flight on the expression of various cell surface antigens. First, we will measure changes in circulating levels of adhesion molecules using specific a specific immunoassay. Second, we will measure cell surface expression of L-selectin, LFA-1, MAC-1 and VLA-4 on leukocytes using flow cytometry. Finally, we will quantify endothelial cell surface expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 using an established, dual radio-labeled antibody binding technique.

All assays will be performed in tissues and cells obtained from anti-orthostatically suspended and control mice, under non-inflamed and inflamed conditions.

3.5 To examine the influence of anti-orthostatic suspension on the inflammatory functions of neutrophils and macrophages.

In addition to recruitment, proper activation of inflammatory leukocytes is critical for effective host defense against invading microorganisms. Consequences of activation include enhanced oxidative metabolism, phagocytosis and bactericidal activity. We will isolate neutrophils and macrophages from anti-orthostatically suspended and control mice, and directly measure their ability to produce reactive derivatives of oxygen, and to phagocytize and kill various microorganisms *in vitro*. These studies will allow us to determine if anti-orthostatic suspension alters the primary, innate immune responsiveness of inflammatory leukocytes, thereby affecting subsequent infectious susceptibility.

3.6 To determine if cytokines and chemokines relevant to the inflammatory process and adhesion molecule expression are altered by anti-orthostatic suspension.

Another mechanism by which anti-orthostatic suspension may influence the inflammatory process is by directly affecting the production of inflammatory cytokines and chemokines. It is known that the intensity, chronicity and type of inflammatory response is largely determined by the production of specific cytokines. The relative ratio of CXC versus CC chemokines appears to determine the nature of the inflammatory response. For example, IL-8, the most important CXC chemokine is responsible for the chemotactic activity and activation of neutrophils. In contrast, RANTES and monocyte-chemotactic protein (MCP) are abundant in mononuclear cell-rich inflammatory foci. Moreover, the expression of specific chemokines is controlled by cytokines, such as IL-1, IL-4, IL-10 and TNF-α that serve as either positive or negative mediators in the regulation of chemokine production, thus controlling the recruitment of leukocyte subpopulations into the inflammatory site. We will study the synthesis of cytokines and chemokines in anti-orthostatically suspended and control mice, in response to nonspecific an specific inflammatory stimuli.

4. Modifications Required and Rationale for Modifications

We began our studies by examining the effect of anti-orthostatic suspension on the development of DTH. A murine DTH model was successfully developed in our laboratory. This model entailed sensitizing mice with oxazalone, a contact sensitizer which appeared to generate more consistent results than the originally proposed chemical sensitizer, dinitrofluorobenzene. This model was successful in our laboratory, therefore, we proceeded to study the effect of anti-orthostatic suspension on DTH. In addition, we directly studied the effect of suspension on leukocyte migration into the peritoneum and activation in vitro.

5. Summary of Progress and Relation of Research Findings to Specific Aims, Hypotheses, and Objectives

We hired and trained a technician to carry out the studies proposed. In addition, we were able to construct 30 cages for the anti-orthostatic suspension of mice. A pilot study was performed to ensure that the cages were appropriate and the mice (C57Bl/6 strain) would survive the 14 day suspension protocol. We used 6 mice to test the anti-orthostatic suspension protocol. At the end of the 14 days, it was clear that the mice were well adjusted to their environment and would survive. The tail suspension apparatus and cages needed a few modifications, which have been implemented. Overall, this study was successful and we proceeded to study the effect of anti-orthostatic suspension on the development of a DTH reaction.

The DTH reaction was induced in anti-orthostatically suspended, orthostatic controls and normal control mice by contact sensitizing and challenging mice with a chemical allergen, oxazalone. Briefly, mice were sensitized with oxazalone (3% solution painted onto shaved backs) on day 8 of suspension. On day 13 of suspension, thickness of both ears was measured using a micrometer, and mice were then challenged on one ear with 3% oxazalone and one ear with acetone (vehicle - control). On day 14 of suspension (24 hours post challenge), ear thickness was again measured. The change in ear thickness (Day 14 - Day 13) was used as an index of ear swelling and the development of a DTH reaction. This is a well established model of assessing the mechanisms of cell-mediated immunity.

Our data demonstrated that all animals responded to antigen challenge and developed a DTH skin reaction, as measured by a change in ear thickness **(Figure 6)**. Furthermore, the DTH reaction in anti-orthostatically suspended mice was significantly greater than both the normal and orthostatic control mice. The anti-orthostatically suspended mice had a relatively greater baseline (Day 13) ear thickness when compared to orthostatic and normal control mice (data not shown). This was expected as it is known that anti-orthostatic suspension induces cephalad fluid shifts. These data were presented at the First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999.

In addition to the DTH study, a number of techniques were established in our laboratory. First, the equipment for intravital microscopy was successfully set up in our laboratory. Intravital microscopy was used for direct visualization of leukocyte-endothelial cell interactions in various vascular beds of anti-orthostatically suspended and control mice. Second, the appropriate licenses were obtained for the use of various radioisotopes, needed to study whole body adhesion molecule expression. We measured baseline ICAM-1 (intercellular adhesion molecule-1) expression in several tissues of normal mice and mice suspended for 14 days. In addition, we measured ICAM-1 expression following systemic endotoxin challenge in normal and AOS mice. Systemic endotoxin induced a significant increase in ICAM-1 expression in all tissues examined in both normal and AOS mice. Furthermore, the endotoxin-induced increase in ICAM-1 expression was significantly higher in the cremaster and abdominal muscles of AOS mice compared to their normal counterparts **(Table 4)**.

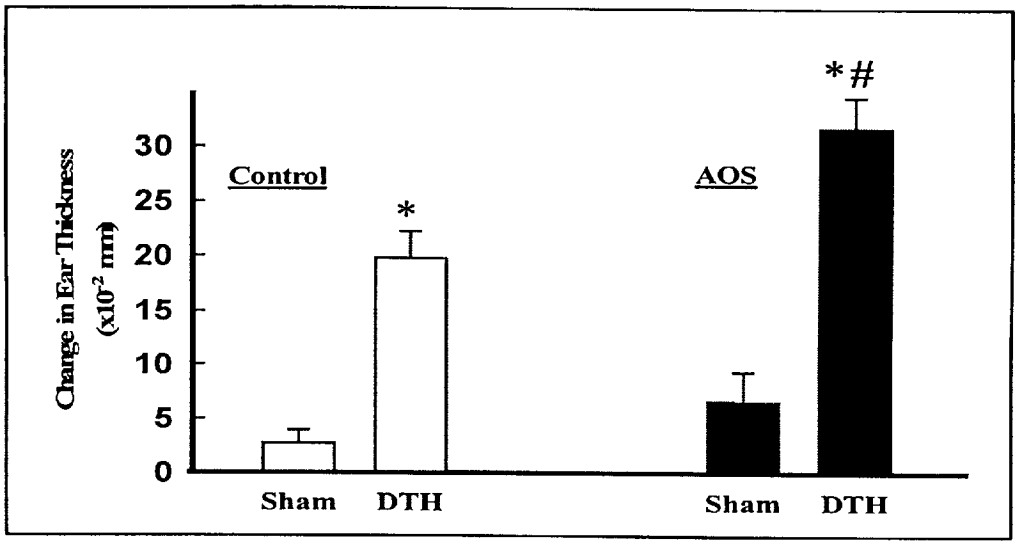


Figure 6: The change in ear thickness, as a measure of DTH, in normal and 14 day suspended mice. Both groups of mice elicited a significant increase in ear thickness. The DTH reaction in AOS mice was significantly greater than in control mice. * $p < 0.05$ relative to respective sham value; # $p < 0.05$ relative to respective control value.

These data suggest that AOS directly influences endotoxin-induced ICAM-1 expression in tissues that are fluid depleted. The consequences of this alteration in ICAM-1 expression are unclear at this point, but are likely to directly influence leukocyte recruitment.

Table 4

Baseline and LPS-Induced (4 hrs) ICAM-1 Expression in Various Organs of Normal and AOS Mice

	BASELINE		ENDOTOXIN	
	Normal	AOS	Normal	AOS
Brain	0.03 +/- 0	0.02 +/- 0	0.17 +/- 0.1	0.15 +/- 0
Heart	0.49 +/- 0.1	0.51 +/- 0.2	1.32 +/- 0.6	3.48 +/- 2
Abdominal Muscle	0.06 +/- 0	0.07 +/- 0.1	0.20 +/- 0.1	0.50 +/- 0.1*
Cremaster Muscle	0.08 +/- 0	0.12 +/- 0	0.10 +/- 0.1	0.90 +/- 0.4*

All values are represented as % injected dose of labeled antibody/g tissue (*$p < 0.05$ relative to respective normal value.

Third, the assays required for *in vitro* assessment of phagocytosis and microbicidal activity of macrophages were established and tested. It became evident that a number of modifications to the original protocol would be required. The studies were carried out and the results were presented in a manuscript which is in press **(See Appendix 3)**. Although the original project had several components, but the basic objective: determine whether biochemical or structural components of the inflammatory system and cellular molecule adhesion system are altered in the AOS murine model of space flight. The underlying hypothesis was that local tissue fluid shifts associated with the AOS model would affect the tissue distribution of adhesion molecules and, therefore, alter inflammatory responses. This hypothesis was partially validated in the experiments to date, in that an enhanced DTH skin test response to a recall antigen was seen in the AOS mouse, and an upregulation of endotoxin-induced ICAM-1 molecules was documented in fluid-deprived muscle tissue.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

A. SPECIFIC AIM 1

Our research project has addressed two very important Critical Research Path Risks: 1) immunodeficiencies/infections (rank 1 [high priority], type III [problem suspected]) and 2) carcinogenesis (rank 1, type III). Our plan was to demonstrate possible harmful effects of known conditions of long-term space flight (stress, isolation, containment, microbial contamination, sleep deprivation). Our progress to date leads us to believe that we should continue this plan for preparation for interplanetary space travel. The unknown effects of microgravity and solar radiation upon the normal balance of immunity needs careful exploration with additional models of space flight. Clinical evidence from patients on Earth show that the immunosurveillance system, when suppressed by virus infection by therapeutics, by radiation, and by stress begins to fail in protecting the host against opportunistic infections, reactivation of latent viral infections, and malignancies. We need to devise a countermeasures program to negate the impact of these immunosuppressive factors on astronauts. Our countermeasures readiness level is 1 or 2 (basic research level). Our sleep-deprivation experiments have suggested that short intermittent naps (2-hour naps every 12 hours) prevent the increased production of plasma sTNF-αRI and IL-6, messengers involved in sleep regulation. In the short-term perspective, these findings also hold importance for a countermeasures program for diseases and conditions on Earth (e.g., the sleep-starved electrical lineman killed because he forgot to wear insulation gloves in touching a 7,500 volt line, Houston Chronicle, September 17, 2000). Our studies would indicate that the sleep-inducing levels of sTNF-αRI and IL-6 became so great in this individual that he had a memory loss that caused his death. In the long-term perspective, the chronic elevation of these and other cytokines is likely to lead to increased infections with viruses with cellular receptors are susceptible to upregulation. Moreover, certain of these viral infections have been implicated in initiating the process of malignant cell transformation.

B. SPECIFIC AIM 2

To date a detailed examination of the effects of space flight on adhesion molecule expression has not been reported. It is known that AOS induces significant shifts in tissue fluid, from the hind end of the animal to the fore end. These changes in tissue fluid are evident as head and neck puffiness in animals, and are coincident with observations made in astronauts returning from space. Although the influence of tissue fluid redistribution on the immune/inflammatory response is not entirely clear, it is conceivable that the microvasculature will be directly affected. Specifically, there may be tissue-specific alterations in adhesion molecules. In fact, we have obtained evidence that endotoxin-induced ICAM-1 expression is significantly enhanced in fluid depleted tissues (cremaster and lower abdominal muscles). This is an important observation in light of the fact that ICAM-1 is a critical adhesion molecule involved in leukocyte migration. It is conceivable that the AOS-induced increase in ICAM-1 expression may translate into enhanced leukocyte infiltration in response to endotoxin. Therefore, the consequences of any alteration in the inflammatory cascade during prolonged space flight may be critical to overall human health.

APPENDICES

1. PROJECT RESEARCH DATA (CONTAINED IN REPORT)

2. LIST OF PUBLICATIONS

a. **Shearer WT**, **Lugg DJ**, **Rosenblatt HM**, Nickolls PM, Sharp RM, **Reuben JM**, **Ochs HD**. Antibody responses to bacteriophage ϕX-174 in humans exposed to the Antarctic winter-over model of space flight. *J Allergy Clin Immunol*, in press.

b. **Shearer WT**, **Reuben, JM**, Mullington JM, Price NJ, **Lee B-N**, **Smith EO**, Van Dongen HPA, Szuba MP, Dinges DF. Soluble tumor necrosis factor-alpha receptor I and interleukin-6 levels in humans subjected to the sleep deprivation model of space flight. *J Allergy Clin Immunol*, in press.

c. **Shearer WT**, Sonnenfeld G. Alterations of immune responses in space travel. In Hans M, ed. Encyclopedia of Space Science and Technology. New York: John Wiley & Sons, in press.

d. **Smolen JE**, Fossett MC, Joe Y, Prince JE, Priest E, **Kanwar S**, **Smith CW**. Antiorthostatic suspension for 14 days does not diminish the oxidative response of murine neutrophils. *Aviat Space Environ Med*, in press.

3. COPIES OF MANUSCRIPTS *(Included with Signed Original Project Report Only)*

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

FINAL PROJECT REPORT

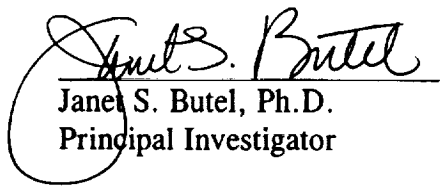
2000

PROJECT TITLE: Immune Function and Reactivation of Latent Viruses

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9/20/00
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EXECUTIVE SUMMARY

A major concern associated with long-duration space flight is the possibility of infectious diseases posing an unacceptable medical risk to crew members. One major hypothesis addressed in this project was that space flight will cause alterations in the immune system that will allow latent viruses that are endogenous in the human population to reactivate and shed to higher levels than normal, which may affect the health of crew members. The second major hypothesis examined was that the effects of space flight will alter the mucosal immune system, the first line of defense against many microbial infections, including herpesviruses, polyomaviruses, and gastroenteritis viruses, rendering crew members more susceptible to virus infections across the mucosa.

We focused the virus studies on the human herpesviruses and polyomaviruses, important pathogens known to establish latent infections in most of the human population. Both primary infection and reactivation from latent infection with these groups of viruses (especially certain herpesviruses) can cause a variety of illnesses that result in morbidity and, occasionally, mortality. Both herpesviruses and polyomaviruses have been associated with human cancer. Whereas normal individuals display minimal consequences from latent viral infections, events which alter immune function (such as immunosuppressive therapy following solid organ transplantation) are known to increase the risk of complications as a result of viral reactivations. As this was a new research effort for the project team, initial efforts included development of dedicated laboratory facilities, training of personnel, establishment of collaborations, and development of assays. Special cages were tested, design modified, and then fabricated for the antiorthostatic suspension (AOS) mouse mucosal immunity studies.

The strategy of this project was to measure the frequency and magnitude of viral shedding from humans participating in activities that serve as ground-based models of space flight conditions. First, however, using sensitive polymerase chain reaction (PCR)-based assays for herpesviruses and for polyomaviruses, we established baseline patterns of virus reactivation and shedding in normal healthy volunteers (n = 30) in a one-year-long longitudinal study. We found that normal individuals over age 40 frequently shed polyomavirus JCV in urine, and some normal individuals shed high levels of herpesvirus EBV in saliva, indicating that viral contamination within a spacecraft is an issue to be considered. We then organized collaborations involving several ground-based human models that mimic certain aspects of space flight. These included wintering-over in Antarctica (collaborators D.J. Lugg and D.L. Pierson); a Russian closed chamber study in which individuals were confined within a space-craft-like chamber on the ground (collaborator I. Larina); a sleep-disruption model (collaborator, J. Mullington); and HIV-infected individuals, a medical condition in which patients suffer immunosuppression due to infection with HIV, the AIDS virus (collaborator, C. O'Sullivan). Analyses of specimens from these space analog models are still in progress, but there is preliminary evidence of increased viral reactivation and shedding, suggesting that space flight conditions can alter the host-pathogen status and result in viral reactivation. These types of data will guide decision-making regarding the necessity of countermeasure development.

We addressed the mucosal immune system questions by using a ground-based mouse model (AOS of mice) and rotavirus (a gastroenteritis virus known to be a mucosal immunogen and to cause human disease). This model system does not simulate all aspects of space flight, but it is accepted as a model for studies on alterations of the immune system. Our results from the AOS mouse model suggest that alterations in mucosal immune responses do occur under simulated space flight conditions, but that neither a delay in rotavirus clearance nor possible alteration of IgG1 anamnestic antibody responses was critical for the resolution of primary rotavirus infection or protection from rotavirus challenge. However, our experiments do not exclude that other important alterations in the mucosal immune system may have occurred. We believe that an examination of more global changes in the mucosal immune system would provide a more thorough cataloging of the effects of AOS on the mucosal immune system.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

"Immune Function and Reactivation of Latent Viruses"

Final Project Report

2000

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I. RESEARCH PLAN SUMMARY

A. HYPOTHESES AND SPECIFIC AIMS

Under most circumstances, latent viruses are held in check by the normal immune system of the host. However, there are indications that crew members of space flight missions may suffer alterations in immune function.

One major hypothesis addressed during this project was that space flight will cause alterations in the immune system that will allow latent viruses endogenous in the human population to reactivate and be shed to higher levels than normal, which may affect the health of crew members (both the individual undergoing reactivation and contacts to whom reactivated virus might be transmitted). The second major hypothesis examined was that the effects of space flight will alter the mucosal immune system, the first line of defense against many microbial infections, rendering crew members more susceptible to virus infections across the mucosa. We focused the virus studies on the human herpesviruses and polyomaviruses, important pathogens known to establish latent infections in the human population. Events which alter immune function (organ transplants, HIV infection, etc.) are known to increase the risk of complications as a result of viral reactivations.

The specific aims we addressed were: (1) To study the frequency and magnitude of reactivation and shedding of latent herpesviruses and polyomaviruses using ground-based human analogs of space flight; and (2) To determine whether the mucosal immune system is adversely affected by space flight using the ground-based animal model, the antiorthostatic suspension (AOS) of mice.

B. APPROACH

There are several ground-based human models that mimic certain aspects of space flight (but not microgravity). One is wintering-over for 8–12 months in Antarctica. As our collaborator, D.J. Lugg, summarized, "The hostile, dangerous and unfamiliar Antarctic environment with its total isolation, cold and photoperiodicity is arguably the most extreme and ... isolated on Earth. The majority of the small, confined groups wintering for up to a year have had to travel great distances to reach their destination, are totally self-sustaining and require complex operations for maintenance. They therefore provide an excellent analog for groups in space." Another space analog is a closed chamber study in which individuals are confined within space-craft-like chambers on the ground. Both these conditions mimic, but cannot precisely duplicate the stress, confinement, isolation, and microbial contamination expected to be encountered during actual space flight. Another ground-based model focuses on the sleep-disruption aspects of space travel. To these we have added HIV-infected individuals, a medical condition in which patients suffer immunosuppression due to infection with HIV, the AIDS virus. HIV-infected individuals are the most extensively studied and perhaps best understood immunocompromised population. They are an effective model of medical problems that arise due to damage to the immune system. By studying patients in various stages of HIV-related disease, different degrees of immune damage can be modeled. Finally, for rodent studies, there is the antiorthostatic, hind-limb suspended,

head-down tilt model that simulates some aspects of weightlessness that occur during space flight. We incorporated all these models into our multi-pronged approach to assessing the possible deleterious effects of space flight on viral infections and disease. Because of the time required to organize the necessary collaborations and the long-duration nature of some of the model systems, we have not completed all the analog studies. When completed, this will represent the first comprehensive comparison, using the same battery of sophisticated assays, of several different ground-based models. It should reveal how these flight analog models agree or differ with respect to immunological and virological parameters.

We chose to examine representative viruses known to be found in saliva, blood, or urine, both because of ease of sample collection from study volunteers and because they raise the possibility of contamination and spread within the spacecraft environment. Herpesvirus EBV replicates in epithelial cells of the oropharynx and parotid glands and virus is present in saliva. It also infects and immortalizes B lymphocytes, although at any given time very few of the lymphocytes release virus particles. Polyomaviruses establish infections of the kidneys that may result in viruria; these viruses also may be present at very low levels in peripheral blood mononuclear cells (PBMCs).

Because of the exquisite sensitivity of the polymerase chain reaction (PCR) and the expectation that only small amounts of virus will be present in human specimens, a laboratory dedicated to this project that is removed from on-going studies was developed to avoid potential problems of sample contamination. Space was provided by the institution and a laboratory was designed to have a dedicated sample processing room and a clean room for PCR assay set-up (from which control plasmids are excluded).

The rat cage design suggested by the original reviewers required modifications for use with mice in biohazard Hepa-filtered racks; the cages were fabricated in the Baylor Surgical Fabrication shop. Several design flaws in the prototype cage had to be modified to allow the mice to easily move the pulley system freely around the cage and to facilitate fecal sample collection (Figure 1).

C. Summary of Results

1. Normal Volunteer Longitudinal Study: Polyomaviruses

A one-year-long longitudinal study of 30 normal healthy volunteers was designed and completed, with our collaborator Dr. W. Keitel (Figure 2). Blood, urine, and saliva samples were collected at 2-month intervals and analyzed by PCR techniques for viral DNAs. This was necessary to provide the first year-long study of reactivation and shedding patterns of herpesviruses and polyomaviruses in normal individuals so that results of virus reactivation studies in ground-based analogs of space flight can be meaningfully interpreted in the context of normal, baseline reactivation patterns.

DNAs extracted from cells pelleted from the urines of the volunteers were tested for the presence of polyomaviruses JCV, BKV, and SV40. PCR was first attempted with primers against a cell gene sequence to test whether the samples could be used for PCR reactions and most

samples appeared suitable. PCR results of DNA extracted from urine pellets and tested for JCV are summarized in the Appendix (Table 1). All urine samples were negative for BKV and SV40 DNA sequences. Using similar methods, none of the blood samples from the 30 healthy volunteers contained detectable levels of polyomavirus DNAs. JCV excretion was detected in urine samples from 14 of 30 (46.7%) subjects (Table 2). Viral shedding was most consistently observed in subjects over 40 years of age [11/17 (64.7%)]. Urinary excretion was more sporadic in subjects <40 years old, and more consistent in older subjects (Table 3). Frequent viral shedders were defined as subjects with 3 or more JCV-positive urine samples out of 6 specimens tested. Eight of nine (88.9%) frequent shedders were over age 40.

Sequence of certain regions of the polyomavirus genome (i.e., viral regulatory region) can be used to distinguish field isolates from one another and from laboratory strains. The regulatory region of JCV isolates from persistently infected kidneys is typically archetypal (lacking large repeated elements within the viral enhancer), whereas the regulatory region of viruses associated with JCV-induced progressive multifocal leukoencephalopathy is complex (rearranged or containing repeated elements within the enhancer). DNA sequence analysis was performed on PCR products from selected samples; the results verified identity (JCV), genetic architecture of the regulatory region (archetypal), and proved the results were not due to contamination with the PCR positive control template DNA which has a complex regulatory region (data not shown). JCV can be subtyped based on the sequence of the V-T-intergenic region (between the ends of the JCV large tumor antigen gene and the late virus coat protein [VP1] gene) or a segment of the VP1 gene. The identification of subtypes is a useful epidemiological tool. To determine the JCV type and subtype shed by normal volunteers, ten specimens were chosen at random for analysis and PCR primers were used to amplify a 215-bp JCV VP1 sequence. Six different subtypes were represented among the ten volunteers. Each virus shedder excreted the same virus subtype in serial samples.

2. Normal Volunteer Longitudinal Study: EBV

EBV DNA was measured in saliva and blood samples from the normal volunteer longitudinal study. These assays were done using a colleague's ABI Prism 7700 sequence detector. Using this instrument, one can detect and monitor target sequences (like EBV) in real time as the PCR takes place. Few studies have attempted to quantitate EBV genome levels in normal individuals. We used the real-time PCR-based assay to detect EBV in ten of the normal volunteers (Table 4). The trend is that individuals vary in the amount of EBV detectable in either saliva or blood. Three subjects shed low levels of virus that were detectable on only one or two occasions over the course of 1 year, two subjects showed detectable virus shedding in saliva on every occasion, and other subjects shed virus more sporadically. It is interesting that the genome copy number of EBV detected in saliva approached 10^5 to 10^6 in some individuals. We speculate that, given these high levels, EBV could be spread easily in closed environments. Unlike JCV, EBV shedding does not appear to occur in an age-dependent manner. In addition, there does not appear to be a correlation between subjects who frequently shed JCV and those who shed EBV (Table 5). This observation reinforces the possibility that different individuals control latent viral infections differently and makes a case for surveying multiple viruses from individuals inhabiting ground-based analogs of space flight. Finally, we observed that EBV DNA levels in the blood from these same normal volunteers never exceeded 100 EBV genome copies/10^5 peripheral blood

lymphocytes (PBLs) (Table 4), whereas HIV-infected individuals, who are at greater risk of opportunistic infections, including EBV-associated lymphoma, often had higher EBV genome levels, sometimes reaching thousands/10^5 PBLs.

3. Ground-Based Model of Space Flight: Winter-Over in Antarctica

We have recently received (in March 2000) from our collaborator, Dr. D. Lugg, specimens of blood, urine, and saliva that were collected during the 1999 winter-over in the extreme environment of Antarctica. Analysis of these samples is in progress. While awaiting the return of the polar expeditioners, we carried out preliminary studies on urine specimens collected during the Antarctic winter of 1998 (provided by our collaborator, Dr. Pierson). JCV excretion was detected in samples from 7 of 30 (23.3%) test subjects, with shedding more frequent in subjects over 40 years of age [4/7 positive subjects (57.1%)] (Table 6). Recalling that no detectable shedding of polyomavirus BKV occurred in the normal volunteers, it is of particular importance that BKV was detected in the urine of 5 of 30 (16.7%) Antarctica subjects (Table 7). These findings suggest that latent BKV infections are reactivated in humans subjected to prolonged cold-stress of the extreme environment of Antarctica. Dr. Pierson observed excretion of CMV by 14/30 (46.7%) expeditioners and by only 2/9 (22.2%) normals (Table 6). If those preliminary trends hold in follow-up studies, it will suggest that an effect of the stress of isolation in Antarctica leads to an alteration in the host-pathogen status as reflected in increased viral reactivation and shedding.

4. Model for Immune Status and Virus Reactivation: EBV in HIV-Infected Individuals

An initial goal of this project was to develop a sensitive PCR-based assay, quantitative competitive PCR (QC-PCR), for detection and quantitation of EBV from clinical samples. This goal was accomplished and an example of data is shown in Figure 3. We utilized this assay to measure EBV genome loads in HIV-infected individuals before and after treatment with highly active antiretroviral therapy (HAART). These samples were obtained through collaboration with Dr. C. O'Sullivan at the University of Alabama, Birmingham. As many HIV-positive patients respond well to HAART and their immune systems rebound, this clinical situation afforded an opportunity to monitor viral loads of an opportunistic, reactivated viral infection (EBV) and attempt to derive correlations with the immune status of the host.

The EBV genome load patterns in the blood at baseline and following HAART treatment were not uniform among all individuals (Table 8); four trends emerged. Some individuals (Patients 1–5) had higher EBV loads initially that tended to decrease over time with treatment. Another group (Patients 6–10) showed a sharp spike of EBV loads shortly after treatment that fell with time. A third group (Patients 11–14) had little detectable EBV until later time points. Finally, some individuals (Patients 15–19) displayed little or no detectable EBV at any time. Despite the variability among individuals, we observed a significant correlation between $CD4^+$ cell counts and EBV load. Those individuals with lower $CD4^+$ counts, a measure of immunosuppression, also had higher EBV genome loads in the blood (Figure 4) [except in the profoundly immunocompromised (<200 $CD4^+$ cells/mm^3), where other factors influence EBV levels]. Thus, the EBV load in the blood serves as a functional marker of immune competence.

We also measured antibody titers to EBV antigens in this same cohort of patients. Antibody titers against EBV antigens produced during active replication of the virus were determined and included IgG-VCA (viral capsid antigen) and IgG-EA (viral immediate early genes). Antibodies detected to these proteins likely represented secondary immune responses to the virus because they were IgG class molecules. Three patients who developed high EBV loads in the blood during HAART treatment showed increases in both anti-VCA and anti-EA titers that were well above those typically observed in normal individuals. IgM titers to VCA were low in all cases, indicating there were no primary infections by EBV. Finally, anti-EBNA antibodies were generally low. (EBNA antigens represent EBV proteins synthesized during the latent phase of the viral life cycle.) We conclude that EBV serology may be a useful measure of changes in the status of EBV replication in immunosuppressed individuals. The antibody titers determined in this cohort of patients with a range in degree of immunosuppression will serve as a useful benchmark for comparison of EBV antibody titers in inhabitants in ground-based analogs of space flight.

5. Synergy Project Collaboration — Sleep Deprivation Study

We were collaborators on a synergy project awarded to Dr. Janet Mullington of the Human Performance Team to examine possible virus reactivation during sleep deprivation. Ten subjects were in the study; five were controls with 8 hours sleep per night and five were test subjects that were partial sleep-deprived (4 hours sleep per night) for 10 days. Samples of blood and urine were collected at the start of the study to determine baseline values and again after the 10th night of sleep deprivation (test values). Samples collected from the ten test subjects were shipped from Boston to Houston under code. The samples were processed and assayed for polyomavirus and EBV DNA sequences, using the same methods as described above. Urine from one subject was positive for JCV at both collection times; other urines were negative for JCV. No BKV or SV40 sequences were detected in urine samples. All the PBMC DNA samples were negative for EBV, BKV, JCV, and SV40 DNA sequences. We concluded that under the conditions of partial sleep deprivation designed for the synergy project, polyomaviruses and EBV were not reactivated.

6. Closed Chamber Study

Attempts to integrate this project with chamber studies at NASA/JSC were delayed due to cancellation of planned chamber studies for 1999. As an alternative, we established a collaboration with Dr. Irina Larina and the Russian space program to monitor viral reactivation during a 240-day chamber study (SFINCSS). The chamber study has been completed and specimens were received in Houston in September 2000 for analysis.

7. Effects of Space Flight Conditions on the Mucosal Immune System

We used AOS of mice to determine whether the mucosal immune system is adversely affected in this ground-based model of space flight. We utilized rotavirus and the well-characterized immune response to rotavirus to address the following specific aims: to determine whether induction of a primary mucosal immune response and clearance of a primary rotavirus infection is altered; and whether induction or an established protective mucosal memory immune response is altered.

8. Effects of AOS on a Primary Mucosal Immune Response and Clearance of a Primary Rotavirus Infection

To determine if AOS altered rotavirus clearance, both short (14 days) and long (28 days) AOS experiments were performed (Figure 5). Primary rotavirus clearance was compared among mice housed under three conditions: normal housing (normal), orthostatic restraint (Ortho), or AOS. Control mice housed normally were inoculated with phosphate-buffered saline (PBS). Normal, Ortho or AOS mice (5/group) were inoculated orally with 10^4 infectious doses 50% (ID_{50}) of EC_{wt} murine rotavirus. Rotavirus clearance was evaluated by daily collection of fecal samples 0–10 days postinoculation (dpi) from individual mice and tested for virus shedding or fecal antibody by ELISA.

In the short AOS experiment, mice were inoculated 4 days following suspension, suspension was maintained for an additional 10 days (total of 14 days), and mice were then returned to normal housing. Control mice were inoculated and housed normally. Mock-inoculated mice did not shed rotavirus (Figure 6). All mice inoculated with rotavirus shed virus beginning 2–3 dpi and shedding resolved by 9–10 dpi. However, the mean duration of rotavirus shedding was significantly longer in AOS mice (n=5, 8.2 days) than Ortho mice (n=5, 6 days, p=0.028) or normal mice (n=15, 6.7 days, p=0.0003) (t test). AOS delayed rotavirus clearance. We determined that intestinal transit time is not altered by AOS. To determine if delayed clearance was due to a delay in induction of fecal antibodies, rotavirus-specific fecal total (IgG, IgM, IgA) and IgA antibody were tested. No significant differences in geometric mean titer (GMT) of either antibody type were observed at 6 or 8 dpi ($p \geq 0.083$, Mann Whitney U). Therefore, the delay in virus clearance caused by AOS was not due to a delay in development of fecal antibodies, but by implication was likely due to a delay in $CD8^+$ T cell responses. To determine whether more profound changes in mucosal immune responses would occur under longer AOS, mice were subjected to 28 days of AOS. Mice were suspended for 14 days, inoculated with rotavirus on day 14, and maintained for an additional 14 days. The 28 day AOS has not been reported for mice, but it was well tolerated. In contrast to results with 14 day AOS, there was no delay in rotavirus clearance in 28 day AOS mice (Figure 6). These results indicate that short (4 day), but not longer (14 day), AOS before inoculation causes a temporary change in mucosal immune ($CD8^+$ T cells) responses which delays virus clearance. This temporary change is likely stress related and mice inoculated 14 days after initiation of AOS may acclimate to the stressors of AOS prior to rotavirus inoculation and can then clear virus normally.

9. Effects of AOS on Induction or an Established Protective Mucosal Memory Immune Response

To model whether crew members exposed to pathogens for the first time in space would respond with normal primary and memory mucosal antibody responses, we determined whether protective mucosal immune responses are initiated and maintained in mice that undergo primary virus infection during AOS. After primary rotavirus infection of normal mice, antibody provides long-term protection from virus reinfection. Serum (total) and fecal (total and IgA) GMT and pooled IgG1, IgG2a, IgG2b and IgG3 titers were determined by ELISA. Mice were challenged with rotavirus at either 45 or 114 dpi for the short or long AOS experiments, respectively. No differences in primary serum or fecal rotavirus-specific antibody GMTs or pooled IgG subclass

titers were observed prior to challenge between any of the groups within either AOS experiment. To determine whether these antibody responses were protective, all mice were challenged with murine rotavirus. In both experiments, virus shedding was detected only in rotavirus naïve mice; no shedding was detected in any mouse previously infected with rotavirus. Therefore, AOS at the time primary antibody responses are initiated and memory immune responses are established do not adversely affect protective mucosal antibody responses.

To model whether crew members first exposed to pathogens on earth would mount a normal mucosal secondary or memory immune response in space, we determined whether protective mucosal memory immune responses established under normal conditions were altered by AOS at the time of rotavirus challenge. Mice were housed normally for primary rotavirus infection and, at the time of rotavirus challenge, mice were placed for the first time under Ortho or AOS conditions. Mice were restrained or suspended for 4 days prior to and 10 days post-rotavirus challenge, or 14 days prior to and 14 days post-rotavirus challenge, for the 14 or 28 day AOS experiments, respectively. No significant differences were observed in protection from rotavirus challenge in either 14 or 28 day AOS experiments. Therefore, once the memory immune response to rotavirus was established, neither short nor long-term AOS altered the ability of the mucosal immune response to protect mice from reinfection. However, the anamnestic serum IgG subclass antibody response was altered in 14 day AOS mice. In contrast to normal and Ortho mice, AOS mice failed to mount an anamnestic IgG1 antibody response following rotavirus challenge. These results suggested that T helper (TH) cell type 2 (TH2) cytokines that direct IgG1 class switching failed to be induced following challenge of AOS mice. Based on our results from non-NSBRI experiments, the lack of anamnestic IgG1 responses would not affect protection because IgG1 and likely TH2 cytokines are not necessary for protection from rotavirus.

10. Significance

We have established reliable assays to detect two major types of viruses that commonly infect humans (herpesvirus EBV and polyomaviruses JCV and BKV). Normal baselines for virus reactivation and shedding were established by a year-long longitudinal study of healthy volunteers. This was a unique effort, as no long-term longitudinal study on normal individuals has monitored two groups of persistent viral infections in parallel. Normal individuals over age 40 frequently shed polyomavirus JCV in urine, and some normal individuals shed high levels of EBV in saliva. These data indicate that viral contamination within a spacecraft is an issue to be considered. BKV was detected in the urine of Antarctica subjects, in contrast to negative results obtained with normal volunteers, as well as elevated CMV shedding, suggesting that the prolonged cold-stress of the Antarctica winter-over affects the virus-host interaction and leads to virus reactivation and shedding. The amount of EBV load in the blood was elevated in many HIV-infected persons and appeared to correlate with the extent of immunosuppression. We believe that virus reactivation and shedding can be used as functional markers of changes in the host immune system, even in the absence of knowing the precise immunological deviation that allows virus reactivation to occur. Finally, it is important to recall that viruses shown to undergo reactivation are able to cause human disease and cancer, especially in immunocompromised hosts.

Our results from the AOS mouse model suggest that alterations in mucosal immune responses do occur under simulated space flight conditions, but neither the delay in rotavirus clearance nor

possible alteration of IgG1 anamnestic antibody responses was critical for the resolution of primary rotavirus infection or protection from rotavirus challenge. Our experiments do not exclude that other important alterations in the mucosal immune system occurred, just that any changes that occurred could not be identified because they were not critical to handling rotavirus infection. A limitation of using individual pathogens to catalog changes in the mucosal immune system is that it would require testing more pathogens than is practicable to identify the majority of changes. We believe that an examination of more global changes in the mucosal immune system would provide a more thorough cataloging of the effects of AOS on the mucosal immune system.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

Space flight-induced alterations in the immune system, if serious enough, would have marked adverse effects on host control of microbial infections. Consequences of altered host immunity could include virus reactivation, infection, and replication. As all humans are infected for life with latent or persistent viruses, those infections will be uninvited travelers on all space missions, and it is prudent to understand the implications of their presence. This project is addressing correlations between immune system deficits and reactivations of herpesviruses and polyomaviruses. It is not feasible to monitor all viruses known to establish persistent and latent infections in humans, but by studying representatives of two important classes of viruses that target different tissues in the body, we believe we are able to get a sense of the effect of space flight conditions on latent viral infections in general. Virus reactivation and shedding results from the Antarctica and Russian chamber studies and from HIV-positive subjects are being interpreted by comparison to the baseline established from our normal volunteer study; preliminary evidence of increased viral reactivation and shedding suggests space flight conditions alter the host-pathogen status and result in viral reactivation. These types of data will guide decision-making regarding the necessity of countermeasure development. Possible countermeasures include vaccines to boost antiviral immunity in astronauts and the use of immune modulators to stimulate components of the immune system to keep viral infections in check. Herpesvirus antiviral pharmaceuticals might be necessary to treat reactivated viruses (there are currently no effective antiviral drugs for polyomaviruses). Precautions might need to be designed and implemented to inactivate and minimize the spread of excreted viruses in the spacecraft environment. These studies need to be extended to additional ground-based models and new technologies applied to address quantitative changes and underlying mechanisms of space-flight-mediated effects on regulation of infectious disease processes, including the development of virus-associated human cancers. The knowledge gained from studies on virus reactivations in these test models will be applicable to earth-bound individuals at risk of suffering similar virus reactivations due to immunosuppression following organ transplantation or cancer chemotherapy and during pregnancy, old age, and AIDS.

Because the mucosal immune system is the first line of defense against many microbial infections, including gastroenteritis viruses and reactivated herpesviruses and polyomaviruses, a diminution of mucosal immune status would be cause for concern. Our goals have been to define changes in the mucosal and systemic immune systems under simulated space flight conditions, to

determine whether any observed changes would pose significant risks to crew members, and to gain basic information necessary for future design and testing of appropriate countermeasures to abrogate detrimental immunologic changes. Using a ground-based AOS mouse model and rotavirus, our preliminary results suggest that the mucosal immune response is altered with short-term simulated space flight conditions. A 2-day delay in virus clearance during rotavirus infection is of limited concern, but a delay in virus clearance for viruses that invade across mucosa to systemic sites, result in latency, or have the capability to induce severe disease, could have profound consequences. A delay in virus clearance might allow a virus to overwhelm the immune system, resulting in increased virus replication, wider dissemination through the body, and appearance of clinical disease. During the course of our studies, we realized that using the outcome of individual pathogen infections to catalog changes in the immune system caused by AOS would require testing of more pathogens than is practical to be able to conclude that a majority of changes had been identified. Previous studies have shown that changes in immune responses detected using one pathogen may not be identified with another pathogen. More basic studies are indicated to catalog global changes in the immune system under simulated space flight conditions.

APPENDICES

A. PROJECT RESEARCH DATA



Figure 1. Special cage for housing mice that are anti-orthostatically (shown) or orthostatically suspended. Mice are able to move freely around the entire cage.

Figure 2

NORMAL VOLUNTEER
LONGITUDINAL STUDY

Sample collections:

0 2 4 6 8 10 12
Months

Samples: Saliva, urine, blood
Study: n = 30; Gender: 15 M, 15 F;
Ethnicity: 21 Wh, 4 Hisp, 3 Bl, 2 As;
Ages: <35, 35–45, >45 yrs; 10 subjects ea.

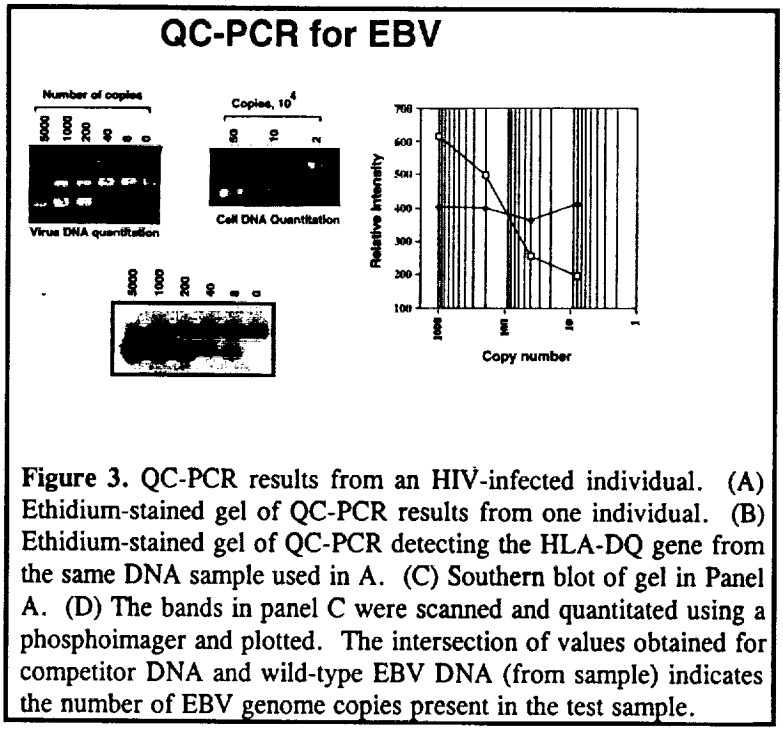


Figure 3. QC-PCR results from an HIV-infected individual. (A) Ethidium-stained gel of QC-PCR results from one individual. (B) Ethidium-stained gel of QC-PCR detecting the HLA-DQ gene from the same DNA sample used in A. (C) Southern blot of gel in Panel A. (D) The bands in panel C were scanned and quantitated using a phosphoimager and plotted. The intersection of values obtained for competitor DNA and wild-type EBV DNA (from sample) indicates the number of EBV genome copies present in the test sample.

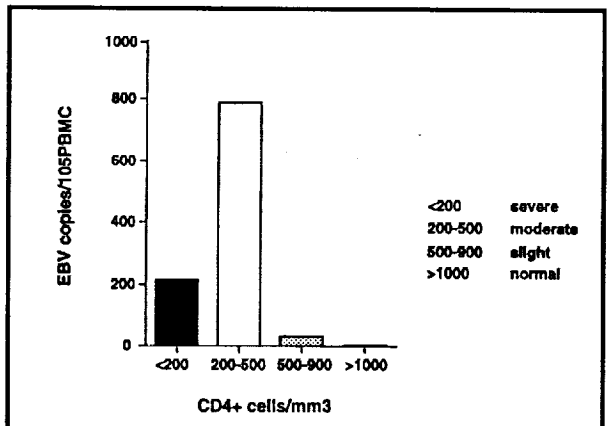


Figure 4. EBV copy number vs. CD4 cell counts.

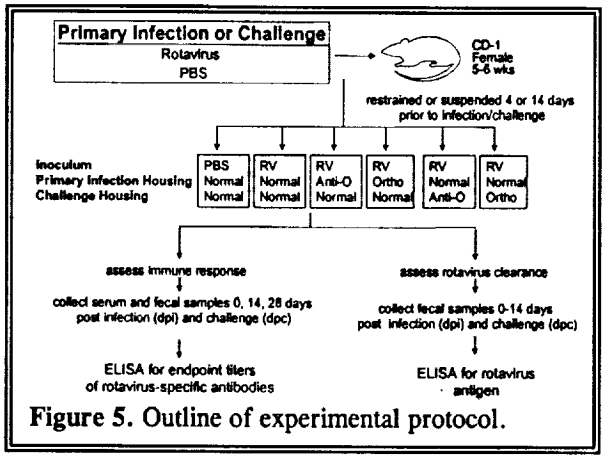


Figure 5. Outline of experimental protocol.

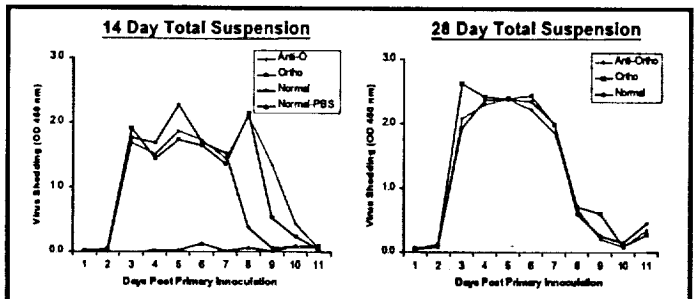


Figure 6. Mean rotavirus antigen shedding following primary rotavirus inoculation in short (14 day) and long (28 day) AOS experiments.

Table 1. PCR of Urine Pellets for Polyomavirus JCV

				JCV[a]						
				1998	*1999*					
Subj. #	*Age*	*Sex*	*Race*	*Nov.*	*Jan.*	*Mar.*	*May*	*Aug.*	*Oct.*	*Dec.*
1	24	F	W	-	-	+	-	-	-	+
2	57	M	W	+	+	+	+	+	+	+
3	31	F	W	-	+	-	-	-	-	-
4	54	F	W	-	-	-	-	-	-	-
5	68	M	W	-	+	-	-	-	NS[b]	-
6	46	M	B	-	+	-	-	-	+	+
7	38	M	L	-	-	-	-	-	-	-
8	30	M	W	-	-	-	-	-	-	-
9	42	F	A	+	+	+	+	+	NS	+
10	42	F	W	-	-	-	-	-	-	-
11	30	M	B	-	-	-	-	-	-	-
12	47	F	W	-	-	-	-	+	+	+
13	43	F	W	-	-	-	-	-	-	-
14	50	F	W	+	+	+	+	NS	+	+
15	53	M	W	+	-	-	-	-	+	-
16	36	M	A	-	+	+	+	+	+	+
17	28	M	W	-	-	-	-	-	-	NS
18	42	M	W	-	+	+	+	+	+	+
19	22	M	A	-	-	-	-	-	-	-
20	24	F	L	-	-	-	-	-	-	-
21	32	F	L	-	-	-	-	-	-	-
22	37	F	W	-	-	-	-	-	-	-
23	42	M	W	+	-	+	+	+	+	+
24	47	M	W	+	+	-	+	+	+	+
25	42	F	W	-	+	-	-	-	-	+
26	24	F	W	-	-	-	-	-	-	-
27	42	M	W	-	-	-	-	-	-	-
28	55	F	L	-	-	-	-	NS	NS	NS
29	56	F	B	-	-	-	-	-	-	-
30	29	M	W	-	-	-	-	-	-	-

[a]+, Detection of JCV DNA by PCR; –, no JCV DNA detected.
[b]No sample.

Table 2. Normal Volunteers — Gender and Age of JCV-Positive Subjects

Grouping		*Fraction positive*[a]	*% positive*
Gender:	Female	6/15	40.0
	Male	8/15	53.3
	Total No.	14/30	46.7
Age:	20–29	1/6	16.6
	30–39	2/7	28.6
	≥40	11/17	64.7
	Total No.	14/30	46.7

[a]Positive at least once out of 7 specimens.

Table 3. Frequent JCV Shedders Are Over Age 40[a]

	Frequent shedders	
Age (yrs)	*Fraction*[b]	*%*
<40	1/9	11.1
≥40	8/9	88.9

[a] Defined as positive ≥3 times out of six collections.
[b]No. positive/no. volunteers tested.

Table 4. Herpesvirus EBV Copy Number in Blood and Saliva

				1*		2		3		4		5		6	
Sub.#	Age	Sex	Race	S[a]	B[b]	S	B	S	B	S	B	S	B	S	B
1	24	F	W	ND	ND	0.004	0.5	59.4	ND	0.09	ND	ND	0.04	0.09	ND
2	57	M	W	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.03	5.2	ND
3	31	F	W	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.01	ND
4	54	F	W	ND	ND	ND	ND	ND	0.9	0.01	ND	ND	ND	ND	ND
5	68	M	W	123	ND	2.15	ND	ND	ND	ND	ND	ND	ND	NC	NC
6	46	M	B	279	0.87	2.41	ND	1.0	ND	146	ND	32.8	ND	11.3	ND
7	38	M	L	0.013	ND	ND	ND	ND	ND	1.38	ND	0.1	ND	0.03	ND
8	30	M	W	0.0006	ND	ND	ND	ND	ND	0.0008	0.4	0.001	ND	0.005	ND
9	42	F	A	0.05	3.85	1.63	6.7	0.0008	17.9	0.01	5.9	0.01	0.5	NC	NC
10	42	F	W	ND	ND	0.001	ND	ND	ND	ND	0.7	ND	ND	0.008	ND

*Collection number; each sample collection was done in 2-month intervals.
[a]Saliva; EBV copy number (×10e4) detected from 500 ng extracted DNA.
[b]Blood; EBV copy number (×10e2) detected from 500 ng PBL (2e5 cells) extracted DNA.
ND, not detected; NC, not collected.

Table 5. Normal Volunteers — JCV and EBV Are Shed Independently

Subject #	*JCV in urine*	*EBV in saliva*
1	2/7[a]	4/6[a]
2[b]	7/7	1/6
3	1/7	1/6
4[c]	0/7	1/6
5	1/6	2/5
6	3/7	6/6
7[c]	0/7	4/6
8[c]	0/7	4/6
9	6/6	5/5
10[c]	0/7	2/6

[a]No. specimens PCR positive/no. specimens tested.
[b]Detection of JCV shedding with little or no detection of EBV.
[c]Detection of EBV shedding without detection of JCV.

Table 6. Antarctica Winter-Over — Gender and Age of JCV and CMV-Positive Subjects[a]

Grouping		*JCV*		*CMV[b]*	
		No. positive[c]	*% positive*	*No. positive[c]*	*% positive*
Gender:	Female	0/6	0.0	3/6	50.0
	Male	7/24	29.2	11/24	45.8
	Total No.	7/30	23.3	14/30	46.7
Age:	20–29	0/7	0.0	1/7	14.3
	30–39	3/12	25.0	7/12	58.3
	≥40	4/11	36.4	6/11	54.5
	Total No.	7/30	23.3	14/30	46.7

[a]Detection of viral DNA sequences by PCR.
[b]Herpesvirus CMV assays performed by Drs. S. Mehta and D.L. Pierson.
[c]Positive at least once out of 4 specimens tested.

Table 7. Antarctica Winter-Over — Gender and Age of Polyomavirus BKV-Positive Subjects

Grouping		*No. positive*[a]	*% positive*
Gender:	Female	0/6	0.0
	Male	5/24	20.8
	Total No.	5/30	16.7
Age:	20–29	0/7	0.0
	30–39	3/12	25.0
	≥40	2/11	18.2
	Total No.	5/30	16.7

[a]Positive at least once out of 4 specimens.

Table 8. EBV Genome Loads in HIV-Infected Individuals Before and After Anti-Retroviral Therapy*

Patient No.	*EBV genome copies/10^5 WBCs*			
	Before HAART	*Time after HAART therapy (months)*		
		1–2	*3–4*	*12*
1	400	160	40	8
2	400	87	–	37
3	327	45	–	ND
4	70	–	–	8
5	40	–	–	0
6	0	667	110	50
7	0	0	128	8
8	167	5000	2449	444
9	85	492	-	87
10	70	200	–	4
11	0	80	50	70
12	4	4	8	851
13	0	1	2	111
14	2	–	40	4166
15	0	0	2	0
16	0	–	0	8
17	0	–	20	3
18	0	4	0	2
19	0	4	–	0

*Each individual is numbered from 1 to 19. Numbers indicate EBV copy number represented as EBV genome copies/10^5 whole blood cells (WBCs). "Before HAART" are baseline samples, and the subsequent time points are months after administration of HAART.

B. LIST OF PUBLICATIONS

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O'Sullivan, C., Peng, R.S. and Ling, P.D. Epstein-Barr virus genome loads in the peripheral blood and antibody titers to EBV antigens in AIDS patients before and after HAART therapy. Manuscript in preparation.

Blutt, S.E. and Conner, M.E. Is the mucosal immune system altered in a ground-based model of space flight? FASEB J. 14:A1202, Abstract 184.13, 2000. Abstract presented at Immunology 2000, The American Association of Immunologists and Clinical Immunology Society Joint Annual Meeting, May 12-16, 2000, Seattle, WA.

Conner, M.E. Determination of whether immune clearance and protection from mucosal virus infection are altered in ground-based mouse models of space flight. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999, League City, TX.

Ling, P.D., Peng, R.S., Pierson, D., Lednicky, J. and Butel, J.S. Latent viruses — a space travel hazard? First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999, League City, TX.

PROJECT TITLE: DNA Probe Design for Pre-Flight and In-Flight Microbial Monitoring

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EXECUTIVE SUMMARY

Crew health is a dominant issue in manned space flight. Microbiological concerns, in particular, have repeatedly emerged as determinants of flight readiness. Microbial infection is prominently featured as a possible risk in the Critical Path Roadmap document (http://criticalpath.jsc.nasa.gov/Risks.asp?DiscMode=D007), and means of monitoring the microbial environment are an important class of countermeasures to be developed. It is essential to the success of long-term missions that systems that deliver acceptable quality of air and water during the anticipated lifetime of the spacecraft be available. As mission duration and re-supply intervals increase, it will be necessary to rely on advanced life support systems which incorporate both biological and physical-chemical recycling methods for air and water as well as provide food for the crew. It therefore is necessary to develop real-time, robust, in-flight monitoring procedures. It would also be desirable if the monitoring system could be readily "reprogrammed" to identify specific pathogens if an in-flight incident were to occur. Thus, the monitoring technology must simultaneously detect many organisms of interest, be subject to miniaturization and be highly automated. The long-range goal of the project was to develop such monitoring systems. In the shorter term, it would be possible to use the technology being developed to obtain a better understanding of the effects of the space environment on microorganisms

Our underlying hypothesis was that the most appropriate target is either ribosomal RNA or the DNA that encodes it. The small subunit rRNA sequence (16S rRNA) in particular has been determined in several thousand bacterial species. Each of these sequences contains short sub-sequences that are widely conserved throughout the data set as well as other sub-sequences totally unique to, and characteristic of, a particular species. This pattern of sequence conservation made it possible to design oligonucleotide hybridization probes that can distinguish individual organisms, or groupings of organisms. Once an appropriate set of target sub-sequences have been identified for a desired assay, any of a variety of formats can be used to implement the assays. Thus, the final assay system may utilize PCR-amplified nucleic acids or, because rRNAs are high copy number molecules, direct detection systems such as chemiluminescence or fluorescence. Both types of assay are compatible with miniaturization and data can be processed automatically or returned to Earth by telemetry. The project objectives included an examination of alternative implementations and development of space craft compatible methods for sample processing.

The major Project Accomplishments were as follows:

1. We initially demonstrated the feasibility of a DNA chip based assay for monitoring of water quality using probes that target 16S rRNA. This result also validated our probe designs for several species of spaceflight interest. It was at the same time clear that three major issues needed to be addressed to make the approach truly useful. These are (1) development of an appropriate set of hybridization probes with minimal cross-reactivity, (2) the development of spacecraft compatible procedures for extracting and purifying the target nucleic acids and (3) increasing the sensitivity and ease of execution of the assay. The work undertaken in subsequent years focused on these issues and considerable progress was made towards

resolving them. This progress was achieved in part through the development of advanced software for probe design, and also through other accomplishments described below.

2. We established specific probes for essentially all the organisms needed to devise an assay system for monitoring spacecraft water quality. This included probes for total bacteria, Gram negative bacteria, enteric bacteria, *Escherichia coli*, *Vibrio proteolyticus*, *Burkholderia cepacia*, and *Acinetobacter*. Several of these probes will also be required for an air analysis system. Several of these probes have now been successfully utilized in multiple assay formats, including molecular beacons as discussed below.

3. We developed compaction precipitation for purifying DNA and RNA. The new technique, which has significant Earthbound spin-off potential, will be particularly useful in developing and possibly in performing spacecraft-based nucleic acid probe assays. A patent application has been filed, and a *Nature Biotechnology* article on the technique generated a high level of inquiries from outside laboratories. The method is being utilized in research on plasmid-based DNA vaccines for HIV, and the email protocols we have sent out appear to be spreading from user to user. The method has the potential for broad use in molecular biology for cloning, sub-cloning, genomics, DNA sequencing, etc. UH has identified a likely licensee for the technology, and as licensing terms are being finalized plasmid miniprep kit design has advanced to the point that the licensee now has packaging mockups for the commercial spin off product.

4. We found that a well-known method of protein purification is also very effective for many nucleic acid separations. Immobilized-metal affinity chromatography (IMAC) is the basis of the ubiquitous six-histidine purification "tag" for recombinant proteins. We hypothesized that chelated metals might also form ligand interactions with the exposed aromatic base nitrogens of single-stranded nucleic acid molecules. Surprisingly, this prospect has not been previously investigated. IMAC proves to be extremely effective at capturing RNA from mixtures with other molecules, and also for stripping primers e.g., from PCR and sequencing reactions. At least some (possibly all) single-base mismatches can be detected, raising the possibility of developing IMAC-based hybridization assays for microbial identification, SNP scoring, etc. A publication and a patent application are in preparation, and the UH licensing office is in negotiation with at least 5 prospective licensees, including the dominant companies in the field.

5. We applied molecular beacons to rapid, low-labor detection of organisms of spaceflight interest. These DNA hairpin probes bear a fluorophore at one end and a quencher at the other. The beacon becomes highly fluorescent when bound to target sequences in an extended configuration. The resulting homogeneous assay also has the advantage of minimal waste generation and reduced danger of cross-contamination, especially when used with amplification methods such as PCR or NASBA. We have converted several

probes for organisms of space flight interest into beacon formats, and demonstrated simultaneous multiplex detection of several organisms using fluors with non-overlapping spectral properties ("colors"). In preliminary results toward highly parallel detection we have also demonstrated the feasibility of arrays of immobilized beacons, in which positive signals are identified by the position, rather than the color, of fluorescence emission.

These results have significant implications for future work. At this stage enough progress has been made in probe design that it would be possible to begin actual instrument development. This should, however, be accompanied by further refinement and testing of the already validated probes. Additional probes can also be designed and validated such that a prototype instrument could examine either air or water samples. Probe design can also now focus on the identification of possible pathogens or otherwise problematic bacteria without preconceived notions of what these organisms will be.

The two main methods under consideration for microbial monitoring are array hybridization and molecular beacon technology. Both are attractive approaches because they might also be usable with samples from blood, urine, and other crew-derived specimens, as well as water and bio-regenerative life-support system samples. This possibility has been further facilitated by the development of space-craft compatible methods for handling samples. Finally, if a hybridization array instrument were developed for microbial monitoring it would also be useable for in-flight studies of global gene expression. This approach might be an excellent way to determine whether key properties such as growth rates, mutation rates or pathogenicity are likely to be affected by the space environment.

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I. PROJECT RESEARCH ACTIVITY

A-1 HYPOTHESES

Ribosomal RNAs contain sub-sequences that are sufficiently characteristic of individual bacterial and fungal species or groups of species such that it is possible to develop hybridization assays to detect various types of microorganisms in space environments.

A-2. OBJECTIVE

To develop an automated hybridization system that can be used to monitor microbial populations and, when necessary, to identify pathogens in air and water samples during long-duration space missions.

A-3. SPECIFIC AIMS

The specific aims of this project were:

Aim 1: To design a prototype set of probes that could be used to simultaneously monitor the levels of total bacteria, fecal coliforms, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* and *Burkholderia* in order to evaluate the quality of water from a microbiology perspective.

It was believed that assessment of water quality was an important first system to examine. By necessity, water will be completely recycled in any long duration mission and its quality must be assured. Water quality also offers an excellent test system for the approach as it is reasonably well defined which organisms are most relevant. Probes to detect the target organisms were successfully designed.

Aim 2: To synthesize and test the probes needed to implement the prototype system using a fluorescence assay. The utility of each probe will be established individually and then verified when all the probes are used together.

Probe design begins with sequence comparisons. Many thousands of rRNA sequences now exist and sequence comparisons must be automated in order to make reasonable predictions for appropriate probes. In some cases unique probes may not be possible and combinations of probes may be necessary to recognize a particular organisms type. Finding apparently unique sequences is not sufficient however; as the rRNAS are highly structured such that the probe target, although unique may not be readily accessible. This difficulty might be overcome in the actual hybridization experiment by denaturing the RNA or by fragmenting the RNA. These approaches are promising but not certain to work and it remains preferable that the probe work without such additional steps. Other possible sources of difficulty exist too. Probes may hybridize with non-target cellular RNAS or DNAs, differences in sequence length and composition can affect strength of signal, and finally, in a system where multiple organisms are being examined

simultaneously, the probes themselves may interact in unexpected ways thereby disrupting results. It therefore is essential that the effectiveness of each probe must be evaluated experimentally and ultimately all need to be examined together. Over the course of the project we examined a larger number of probes and ultimately obtained at least one and frequently more than one useful probes for every target grouping except *Enterococcus*.

Aim 3: To test a ground-based implementation of the probe design using a hybridization array technology.

It is essential that the actual system to be used in space can be fully automated with results either interpreted directly or returned to Earth by telemetry. Given the current state of technology, it at the outset of the project it appeared that the best way to do this was by using automated medium density (100-500 probes per array) hybridization arrays. The project goal was to determine if it would be feasible to use such arrays with 16S rRNA targeted probes. This goal was met. During the course of the project publications appeared which established that emerging molecular beacon technology was also very attractive. Beacons are fluorescent probes that emit a strong signal as a result of a major structural change that is induced by binding of the target RNA to the probe. The reaction can be monitored in solution using samples that have been only partially purified. We speculated that beacons might also be adaptable to use in an array format. Because beacon studies require far less capital we examined this technology in some detail. We showed that beacons could in fact be successfully used with rRNA to detect organisms of spaceflight interest. We also developed multiplex detection using beacons of differing emission wavelength ("color"), and demonstrated the feasibility of highly-parallel beacon detection using an array format in which positive signals are identified by position, rather than color.

Aim 4: To evaluate and develop appropriate sample preparation procedures.

Samples of air and water are readily collected in the space environment using filtration. A critical issue is the preparation of RNA or DNA (if a PCR protocol is ultimately used) from such samples without the use of toxic substances such as phenol and ethidium bromide and/or energy intensive techniques such as high-speed centrifugation. It therefore would be useful to have non-toxic, energy-efficient means of sample preparation and cleanup suitable for use in the spacecraft environment. This is also an area with tremendous spin-off potential for applications in molecular biology, genomics, genetic diagnostics, etc. Progress in this area was especially good. Three techniques were explored: boronate affinity, compaction precipitation, and immobilized-metal affinity. The latter two were extended for nucleic acid purification by us in the course of this work, and each is now very likely to be patented and commercialized. Precipitation of DNA by compaction agents was known from the literature, but we found that it can serve as the basis of a selective separation of RNA from DNA and vice versa. Immobilized-metal affinity is well-known as a protein purification tool, but we have found that it is also useful as the basis of separation of (partially) single-stranded from double-stranded nucleic acids, allowing applications such as RNA isolation, plasmid purification from alkaline lysates, PCR and NASBA primer cleanup, and possibly hybrid-mismatch detection. In addition to being directly useful in developing and performing probe assays for monitoring microorganisms, these tools may well find broad spin-off applications.

Aim 5: To develop and evaluate additional probe sets for other in-flight applications.

If the water analysis system is shown to be feasible the next priority would be a general system for air. This should be of comparable difficulty to the water analysis system as in general the greatest concern is the level of organisms and a rather general characterization of the populations. Systems to identify pathogens, whether expected or not, are technically far more challenging. Since the water analysis system was not completed we did not have time to begin with the air analysis system. Using funding from another source we were able to demonstrate the feasibility of designing a system to genetically characterize a problem organism.

Progress Aim 1: Probe Design.

A variety of eventual assay formats are possible. In each case however it will be necessary to have probes that are specific for each grouping of interest. Additional probes might also be needed. For example, in a sandwich assay a surface capture probe would be needed to selectively remove the target RNA from total RNA. Specific detector probes would subsequently be used to provide a labeling group and thereby determine if a particular RNA is present among the RNAs that have been captured.

Initially, two primary methods were to be used to design probes depending on the number and nature of the organisms to be detected and those to be excluded. In the first approach, we obtain sets of aligned sequences from the public Ribosomal Database Project (RDP) database (http://www.cme.msu.edu/RDP) which included the target species (or genus) and all closely related organisms. These sequences were then placed into a sequence editor (GDE- Genetics Data Environment) that allowed the sequences to be discriminated according to their identity with the target species. The color capabilities of the program made it easy to visually identify sequence regions likely to distinguish the target species from other organisms. The most promising probes were then searched against the entire 16S rRNA database for spurious matches.

In some instances, e.g. when designing global probes that target any Bacteria to the exclusion of Archaea or Eukaryotic microorganisms, the size of the data base to be considered required a more formal approach. Initially, two computer programs: *find_probe* (a flow is provided in Appendix A) and *most_mismatch* were coded for this purpose. A reference organism, for which complete 16S rRNA sequence data is available, is first chosen e.g., *E. coli*. Next all 13-mers (patterns) from the reference sequence different by at least two bases from all archaeal sequences were identified with *find_probe*. The choice of 13-mers is based on the fact that this is the minimal probe length that is used in standard hybridization experiments. The patterns are next sorted by increasing number of sequences in the target group, in which they were found, using a second program known as *most_mismatch*. The pattern found in the most sequences was kept as a possible probe target, and the sequences in which it was not found were extracted into a new file. The file of patterns was sorted again with *most_mismatch* using this new file, and a second target region then identified. The probe sequences for these two target regions are the complementary sequences from the targets.

Finally, a hybrid of the two approaches worked well for genera specific probes. All the 13-mers in a representative sequence in the target grouping that different from all other genera in at least 2 positions were initially identified with *find_probe*. The sequence regions associated with each promising 13-mer were next visualized in the sequence editor, GDE, to see how these probe regions behave within the target group and the extent to which they can be usefully extended to greater lengths.

In order to simplify and improve the redesign of probes, a new bioinformatics approach was developed during the later stages of the project that more directly searched for unique and characteristic sequences in the ribosomal RNA databases. Known as OligoSCAN., this program selects an oligonucleotide of a fixed length in the target RNA, starting at the 3'end of each sequence and scans it against the rest of the sequences for possible similarities. Only oligomers that do not match any region of other rRNA sequences, in either orientation are selected. The program then selects the next oligonucleotide on the sequence. The program continues to scan each target sequence until the end of the rRNA is reached. A mismatch value can also be specified, in which case the oligonucleotide is considered unique even if it matches regions of other sequences, the total number of matches being less than the mismatch number. The resulting oligonucleotides are concurrently subjected to the Tm analysis, GC content, which eliminates oligonucleotides that are not within a specified range. The program outputs the results in a tabular form, which includes the unique oligonucleotide sequence, the position where it is found. A listing of the source code of OligoSCAN is provided in Appendix A.

OligoSCAN was run with the length of the desired oligonucleotide set at 17 and the mismatch number ranging from 10 - 14. At least one unique oligonucleotide was identified for each sequence in the data set when the mismatch number was 12. The potential oligonucleotides that were identified were crosschecked for specificity against the rRNA database with the well-known BLAST program. By using this approach or the earlier approach we were able to successfully identify candidate probes for all of the groupings that were considered useful for use in routine monitoring of water quality from a microbiological perspective. This included probes for total bacteria, fecal coliforms, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus* and *Burkholderia.*

Progress Aim 2: Probe Testing

The primary objective here was to validate probes, which had been designed for a prototype assay system for rapid examination of water quality from a microbiology perspective. The system was to include probes for (1) total bacteria, (2) enteric organisms as a group- i.e. as a measure of fecal contamination, (3) specifically detect *E. coli*, (4) detect the genera *Enterococcus* and *Burkholderia*, (5) detect the presence of *Pseudomonas aeruginosa* and closely related organisms. Before assembling a multiple probe set, it is essential to ascertain that each individual probe can recognize its target organism(s) and distinguish its target group from the most common interfering organisms. In order to conduct this validation we therefore extracted total RNA from representative non-pathogenic strains of various organisms. Cultures (50 ml) of each bacterial strain were grown and harvested by centrifugation. The cells were lysed and RNA isolated by phenol/isoamyl alcohol extraction procedure and purified by ethanol precipitation. The pellets

were resuspended and stored at -20°C. Alternatively, ribosomes were isolated by a low speed centrifugation followed by a high-speed centrifugation. Ribosomal subunits were then fractionated on sucrose gradients and as required purified rRNA was obtained by phenol extraction of the 30S subunits.

Although several methods were tried for probe validation, it was found that the most effective approach was Northern blotting with ^{32}P labeled oligonucleotides. Individual probes were tested against a panel of RNAs encompassing the types of organisms that should be or should not be detected by the probe under consideration. Thus, for example, a valid probe for *E. coli* was required to not hybridize with closely related enterics such as *Proteus vulgaris*, *Proteus mirabilis*, *Serratia marscens*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*. In many cases, a number of probes had to be tried in order to get one that produced reasonable results. Indeed, in some cases probes that had been published in the literature did not perform as expected. Another interesting result was obtained with probe E3, which was expected to be *E. coli* specific but in fact proved to be the best general purpose Gram-negative detector that we have found to date.

In the case of individual genera the usual problem was distinction from closely related genera. For example, known strains of *Burkholderia* are closely related to *Neisseria* and *Ralstonia*. No detector probe pair was initially found that could distinguish *Burkholderia* uniquely from these other two. Therefore, two pairs were designed; one distinguishes *Burkholderia* from *Ralstonia* whereas the other distinguishes *Burkholderia* from *Neisseria*. It was also appreciated, however, that the fine distinction is not actually critical. A probe which indicates the presence of "*Burkhoderia* like" organisms and which therefore might include both *Burkholderia* and *Ralstonia* will be fine for the intended purpose.

In the end, multiple validated probes for all the targeted groupings were obtained, see Appendix A, except for Gram negatives as a whole where only one validated probe has been found and for the genus *Enterococcus* where none have been found. In view of the continued lack of success with this later grouping, we believe it may be best to instead seek a probe that detects all aerobic low G-C Gram-positive bacteria that would include *Enterococcus*, *Bacillus*, *Lactobacillus*, *Streptococcus* and some rarer genera. Detailed studies in which the various probes are used simultaneously were originally planned but not completed due to the fact that probe validation was more time consuming than anticipated. Preliminary results with an initial set of preliminary probes did not, however, reveal any problems with cross-reactivity. We therefore think it likely that when the entire set of validated probes is complete it will be possible to show that cross reactivity between the probes is not a problem. In order to extend the validated probe set to also include analysis of air samples, it would be necessary to obtain three additional Gram-positive probes and 1-2 probes for key fungal species.

Progress Aim 3. Array Hybridization Assays

A. DNA Chip Arrays. Although the development of monitoring assays in an array hybridization format is considered to be very promising it was not an actual project goal to implement such assays, which would require substantial additional equipment and resources. Rather, the objective was to evaluate the feasibility of using such arrays. This was accomplished in collaboration with

Genometrix Inc. a "DNA chip" company based in The Woodlands, TX. We provided Genometrix with RNA samples from a variety of organisms as well as sequences for capture and detector probes. Capture probes were chemically synthesized by a commercial vendor with a terminal amino group which allowed coupling to epoxide-derivatized glass. The detector probes were synthesized with a terminal digoxigenin group. An enzyme-linked fluorescent assay (ELF-Molecular Probes, Inc.) was used in the initial work. Digoxigenin is used as the recognition hapten and using anti-digoxigenin conjugated alkaline phosphatase, the ELF substrate is cleaved to yield a fluorescent insoluble precipitate, which accumulates at sites where detector probes bind. Genometrix Inc. constructed medium density arrays containing the designed capture probes. These were deposited under mildly basic conditions on glass slides using robotics. Each slide contained 14 copies of a 16-probe array, which was challenged with a number of RNA samples including one unknown. Detection was accomplished using their proprietary CCD proximal detector. Sample results are shown in Appendix A. These initial studies were greatly restricted by the limited number of useable probes then available to us. Nevertheless no significant problems were encountered in conducting an assay in this format. These experiments demonstrated the feasibility of the assay in a DNA chip format and gave us significant preliminary information on the effectiveness of the individual probes.

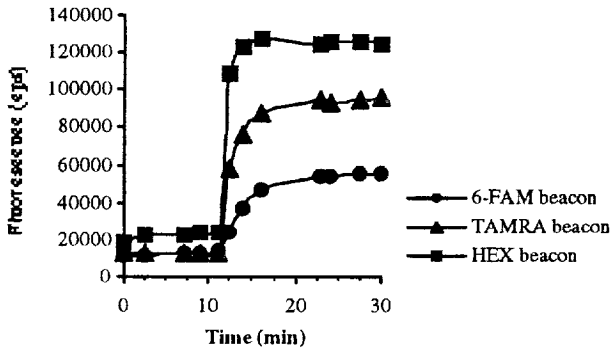
B. Molecular Beacons. Molecular beacons are a recently developed method of homogeneous nucleic acid hybridization assay. The beacon includes a sequence complementary to the target sequence, flanked on each side by ca. 6 bases capable of weak intramolecular hybridization to force the formation of a stem-loop "hairpin" structure. A fluor is covalently attached to one end (in our case, the 5' end for synthetic convenience) of the oligo, and an aromatic quencher is coupled to the other (3') end.

In the absence of target, the beacon self-hybridizes to the hairpin structure, bringing the fluor and quencher into juxtaposition. The fluor is quenched by non-radiative processes which are strongly dependent on distance. In the presence of target, the central probe bases of the beacon (ca. 15 bases) hybridize to the target, forcing the beacon into an extended conformation. Fluorescence is de-quenched, and the target is detected without washing, separation, etc. in a manner potentially very compatible with spacecraft applications.

We have constructed molecular beacons based on probe sequences designed and validated using conventional hybridization assays as described above. Such beacons have generally been functional. For example, the specificity of a 6-FAM labeled molecular beacon directed against *V. proteolyticus* 5S rRNA (sequence 5'- CACGG-TAG CCG CAG CTC G- CCGTG-3') was tested with a variety of phylogenetically different bacterial strains commonly found in contaminated water. Each rRNA sample (3µg total rRNA) in hybridization buffer (20mM Tris-HCl, 10mM KCl, 5mM $MgCl_2$, pH 8.0) was denatured in the presence of molecular beacon (2.3 pmoles) in 50 µl hybridization buffer at 90°C for 5 minutes. After cooling and hybridization, fluorescence was read at an excitation wavelength of 495nm, emission wavelength 510nm, bandpass 9nm. There was a 9.7-fold enhancement in fluorescence when the beacon was hybridized to its target, *V. proteolyticus* 5S rRNA, whereas the fluorescence was close to background when the beacon was exposed to non-target rRNAs from *P. aeruginosa, Enterobacter* sp., B. *cepacia, Acinitobacter* sp. and *E. coli.*

In another test, RNAse H cleavage was used to examine the specificity of a molecular beacon based on a universal probe sequence for eubacterial 16S rRNA (labeled with TAMRA, excitation 528nm; emission maximum 580nm). The beacon's complimentary DNA and RNA targets were used in the experiment. 600µl of hybridization buffer (20mM Tris-HCl, 10mM KCl, 5mM $MgCl_2$, pH 8.0) containing the molecular beacon (58nM) was added to the cuvette and the fluorescence intensity was measured at 45°C. First the RNA target was added in excess (185nM) and the final fluorescence signal was recorded. Then RNase H (Epicentre Technologies, *E. coli*, 40U) was added to cleave specifically RNA strands in RNA:DNA hybrids. The fluorescence of the sample decreased to within 8% of the initial fluorescence level of the unhybridized probe, suggesting that the increased fluorescence was due to the formation of the expected specific beacon:RNA hybrids. A DNA oligonucleotide complimentary to the beacon was then added (184nM) and the fluorescence was restored to the same level as when the beacon was hybridized directly to DNA target without prior RNA/RNase H treatment.

Multiplex detection. In many practical applications, it will be desirable to detect multiple targets simultaneously. As illustrated in the figure below, we have developed simultaneous multiplex beacon assays for multiple targets, based on probes developed as described above and validated in conventional solid-phase hybridization assays, using fluorophores with non-overlapping fluorescence spectra. As described below, we will pursue further beacon development in the proposed continuation work, including the development of beacon arrays theoretically capable of detecting scores of targets simultaneously, without washing and using a single set of excitation and emission wavelengths.



Multiplex simultaneous detection of DNA oligonucleotides in solution using molecular beacons. Three molecular beacons were designed based on probes which target bacterial 16S rRNA. The 6-FAM-based beacon is specific for *Vibrio proteolyticus* (labeled with 6-FAM, excitation wavelength 495nm and emission wavelength 510nm), the HEX beacon is specific for *Burkoderia cepacia* (labeled with HEX, excitation wavelength 526nm and emission wavelength 536nm) and the TAMRA beacon consists of a universal probe sequence for eubacterial 16S rRNA (labeled with TAMRA, excitation wavelength 528nm and emission wavelength 580nm). Their complimentary DNA targets were used in the experiment. 600µl of hybridization buffer (20mM Tris-HCl, 10mM KCl, 5mM $MgCl_2$, pH 8.0) containing the mixture of the three molecular beacons (6-FAM beacon: 28.nM, HEX beacon: 34nM, TAMRA beacon: 44.3nM) was added to the cuvette and the fluorescence intensity for each was measured at 45°C (using the

optimal excitation and emission wavelength for each fluorophore). Then a mixture containing the three complimentary DNA targets in excess was added (6-FAM beacon target: 269nM, HEX beacon target: 110nM, TAMRA beacon: 130nM) and the fluorescence changes followed.

Progress Aim 4. Sample Preparation

We conducted significant exploration of simplified approaches to sample preparation for microbiological and other assays to be conducted in the spacecraft environment. The demands of space flight require that one use the minimum number of steps with minimum manipulation, without employing hazardous materials or excessive energy. We found two of the methods we developed to be quite promising, and each has a high probability of becoming a widely-used spinoff product of this research.

Compaction precipitation. Compaction agents are small, cationic molecules which bind in either the major or minor grooves of a double-stranded DNA or RNA molecule, reducing by four to six orders of magnitude the volume occupied by the DNA or RNA. Compaction agents function *in vivo* to package genomic DNA into sperm (e.g., spermine, spermidine), and can also serve a similar function in the delivery of DNA pharmaceuticals.

In the course of experiments on the potential of nucleic acid compaction for enhancement of chromatographic adsorption capacity (increasing the capacity of classical adsorbents for RNA sample preparation), precipitation was noted at low ionic strengths. Compaction of DNA involves charge neutralization in combination with stabilization of inter-helix interactions. The compaction agent binds in either the major or minor groove, in direct contact with the negatively charged phosphate groups. Precipitation occurs when adjacent DNA helices are affected simultaneously, with the compaction agent not only reducing the helix-helix repulsion but also bridging the helixes. This phenomenon was described in 1981 by Hoopes and McClure, who used spermine to precipitate large DNA molecules from smaller DNA oligonucleotides. Upon further investigation, we observed that RNA is far less readily precipitated by compaction agents, and found that selective DNA precipitation by compaction agents could be useful in purifying plasmids and potentially RNA. A single precipitation step precipitates plasmid DNA quantitatively, leaving nearly all the RNA behind in the supernatant.

We then extended the technique to the isolation of RNA from bacterial lysates using compaction agents such as hexammine cobalt and spermidine. Using 3.5 mM hexammine cobalt, total RNA can be selectively precipitated from a cell lysate and at a concentration of 2 mM hexammine cobalt rRNA can be fractionated from low molecular weight tRNA and mRNA. Using a second stage of precipitation at 7.1 mM hexammine cobalt, the low molecular RNA weight fraction can be isolated by precipitation. The resulting RNA mixtures are readily resolved to pure 5S and mixed 16S/23S rRNA by nondenaturing anion-exchange chromatography. Compaction precipitation was also applied to the purification of an artificial stable RNA derived from *E. coli* 5S rRNA and to isolation of an *E. coli* expressed ribozyme.

The new technique, which has significant Earthbound spin-off potential, will be particularly useful in developing and possibly in performing spacecraft-based nucleic acid probe

assays. Provisional and non-provisional patent applications have been filed on the technique and a *Nature Biotechnology* article on the DNA purification aspect of the technique generated a high level of inquiries from outside laboratories. The technique has been adopted for sample preparation at China's largest DNA sequencing center and is being utilized in research on plasmid-based DNA vaccines for HIV, and the email protocols we have sent out appear to be spreading from user to user. The method has the potential for broad use as a kit in molecular biology labs where enormous efforts and costs are devoted to purifying plasmids for cloning, sub-cloning, genomics, DNA sequencing, etc. UH has identified a likely licensee for the technology, and as licensing terms are being finalized plasmid miniprep kit design is well advanced, to the point that the licensee now has packaging mockups for the commercial spinoff product.

Immobilized-Metal Affinity Chromatography (IMAC) of Nucleic Acids. IMAC is the basis of the ubiquitous six-histidine purification "tag" for recombinant proteins. Based on the chemical similarity of the aromatic nitrogens of nucleic acid bases to those of histidine, tryptophan, etc., we hypothesized that chelated metals might also form ligand interactions with the exposed bases of single-stranded nucleic acid molecules. Literature search and talks with leaders in the IMAC area, (including the inventor of IMAC, J. Porath) establish that, surprisingly, this prospect has not been previously investigated.

To avoid phosphate interaction only soft metals were evaluated, so we expected that single-stranded regions with exposed bases would be favored over double-stranded molecules. This expectation was realized; plasmid, genomic DNA (and lipids and nearly all proteins) have virtually no binding affinity, while RNA and single-stranded oligonucleotides bind strongly to metal-chelating matrices. Interestingly, affinity differs sharply for differing metal ions, decreasing in the order copper(II), zinc(II), nickel(II) and cobalt (II). IMAC proves to be extremely effective at capturing RNA from mixtures with other molecules, and also for stripping primers from e.g., PCR and sequencing reactions. At least some (possibly all) single-base mismatches can be detected, raising the possibility of developing IMAC-based hybridization assays for microbial identification, SNP scoring, etc. A publication and a patent application are in preparation, and the University of Houston licensing office is in negotiation with at least 5 prospective licensees, including the dominant companies in the field.

Progress Aim 5: To develop and evaluate additional probe sets for other in-flight applications.

The immediate goal of analysis of air samples as well as water samples can be accomplished by the addition of additional probes to the validated set. These should likely include a general purpose probes for aerobic low % G+C and high % G+C Gram positive bacteria, individual genera probes for *Micrococcus* and *Staphylococcus*, and fungal probes for *Aspergillus* and *Penicillium*. Sufficient sequence data is already available to design such probe. It is anticipated that an expanded set of fully validated probes could then be used in a single instrument capable of examining both air and water with a single hybridization array.

The more general problem of interest is the identification or genetic characterization of an organism that unexpectedly causes a problem during space flight. Ideally the same instrument should be capable of identifying such a problem organism. If one could anticipate what the problem organism might be this could be approached by further expansion of the set of validated probes. In actual fact the problem organism may potentially be anything.

Insufficient funding was available was to address this issue as part of the NSBRI Funded project. We were however fortunate to have funds from another source that allowed us to explore the feasibility of doing this. It had previously been observed that each rRNA sequence contains particular subsequences that are signatures of the various groups the organism belongs too.We quantified this concept and analyzed 929 16S rRNA sequences to determine the distribution of possible signature sequences. Our results unequivocally demonstrated that enough signature oligonucleotides do in fact exist to readily determine the phylogenetic position of most bacteria. Thus, it is likely possible to determine the closest known relatives of most unknown organisms in a single hybridization array experiment conducted on PCR amplified DNA from the unknown organism.

IMPLICATIONS FOR FUTURE RESEARCH

Traditional Earth based experience alone suggests that pre-flight and in-flight microbial contamination and infection might pose a significant barrier to successful Human Exploration and Development of Space. Such concerns are greatly enhanced by recent studies suggesting that the immune system may be suppressed under space flight conditions, making astronauts more subject to infection. In addition, space flight may have undesirable effects on the microorganisms themselves, including changes in bacterial growth and antibiotic resistance. For example, although no direct studies of bacterial virulence have been made in space, it has recently been shown that modeled microgravity is a novel environmental signal affecting *Salmonella enterica* virulence. It is thus not surprising that microbial infection is prominently featured as a possible risk in the Critical Path Roadmap document (http://criticalpath.jsc.nasa.gov/Risks.asp?DiscMode=D007), and means of monitoring the microbial environment are identified as an important countermeasure to be developed. Among the microbial issues that need to be addressed the following are especially prominent.

A What specific infectious agents will crew-members be exposed to and what are their sources?
D. What diagnostic and environmental monitoring capabilities ultimately need to be developed?
C Do space flight conditions alter growth rates, mutation rates or pathogenicity of microorganisms?
D. Do unique environmental factors inside the spacecraft promote the transmission and activity of microbial pathogens, or caused increased risk of infection, independent of immune function?
E. What countermeasures could be developed to address microbial concerns?

Although ground based research can do much to develop a foundation for answering these questions, it is very clear that space-based experience will eventually be needed. This will necessarily begin with the ability to identify organisms and to process samples while in the space environment. As described below, the work completed here is directly relevant to developing precisely these capabilities and therefore speaks towards answering many of these key microbiology questions.

Instrument Considerations

It is our view that for the foreseeable future there will only be a minimal arsenal of scientific instruments available for use in in-flight research. By necessity, the types of biological instruments that are likely to be available will be those which can be of use in a wide variety of projects. It seems clear that one of the most valuable tools would be a general-purpose hybridization instrument. Such an instrument is potentially useable in assays involving RNA, DNA, proteins and even antibodies and would therefore have a wide range of applications. These might include life detection experiments on the surface of Mars, monitoring changes in the immune system, or detecting microorganisms as discussed herein. A second instrument of great merit would be a PCR/NASBA machine for amplifying DNA which would be invaluable in increasing the sensitivity of rRNA based assays when done in some formats. Thus, we developed the technologies described here in formats that can be readily adapted to instruments of either type. In addition, on some missions such sophisticated biological instruments might not be available. In these instances it will still be possible to examine microbial ecosystems with the molecular beacon technology we have been developing. Our approaches to sample preparation are general and will be useful with any of the likely assay formats. Indeed, this technology appears to have applicability well beyond the isolation of rRNA. For example, compaction agents also provide a powerful method for purifying DNA, which might be of use in non-microbiological work that needs to be conducted by other space scientists.

We have made sufficient progress in developing the specific probes needed for the prototype water analysis system that initial instrument development can begin. It therefore would be timely for the Technology Development Team to begin examining possible instrument designs. Meanwhile, further testing of the existing probe set on mixed samples and unknown samples needs to be completed. In addition it will be necessary to develop useful Gram positive probes. It is also noteworthy that several of the key probes needed for analysis of air are already in place.

Pathogen/Problem Organisms Identification

Our primary focus has been development of real-time, robust, in-flight monitoring technology to determine the levels and general composition of the normal microbial ecosystem. This reflects the fact that it is widely understood that the number one countermeasure against microbial pathogens or microbial caused problems, e.g. build up of biofilms, is to keep microbial levels at reasonable levels. In the long term, this monitoring capability will be used to insure that the spacecraft environment is safe. In the short term these same tools can be used (on the Space

Station for example) to better understand the microbial ecosystems found in spacecraft and how they change over time so that we know what is actually required to maintain a safe environment.

The routine microbial monitoring system described above is not designed to detect specific pathogens. This is not an oversight as such pathogens, if present, would usually have to be sought in clinical samples to be found. Moreover, in the event of microbial infection, the initial countermeasures are largely clear: administer whatever antibiotics are available. There is, however, a concern that a persistent microbial problem might arise from unanticipated directions because of either changed behavior or mutation of a known and usually non-problematic organism. It is uncertain what effect, if any, long-term exposure to the space environment has on bacterial pathogenesis. There is, nevertheless, evidence that relevant bacterial processes such as secretion, adherence, antibiotic susceptibility, etc. are affected by exposure to the microgravity environment. It is therefore entirely possible that during a long duration mission, unanticipated bacterial problems may occur involving organisms that are not normally pathogenic or problematic to space-craft operations. In the event that an unexpected bacteriological problem arises and persists during a long term mission, it will be essential to determine as closely as possible, the genetic identity of the organism that is causing the problem as this will clarify where the organism came from, what treatments are likely to be effective, etc. It therefore would be of value to have a diagnostic system that could readily identify any bacterium that is present regardless of prior expectations of what might be found, so as to facilitate a rapid assessment of what is occurring. Although considerable further research will be needed, we have already shown in work funded by another source that it is feasible to develop arrays of probes to accomplish this task using essentially the same analytical and sample preparation procedures as are proposed for routine monitoring.

Molecular Beacons

Molecular beacons are an exciting alternative because they can in principle be used in simple solution assays, obviating the need for complex instruments while at the same time providing very specific identification of organism types and numbers. Others and we have demonstrated simultaneous detection of 3-5 targets using fluors with differing spectral populations, on beacons in a mixture. Although it appears unlikely that this strategy will be applicable beyond ca. 10 targets it is clear that a useful number of organisms or organism groups can be readily monitored. Thus by using 2-3 separate reaction tubes astronauts will be able to conduct surprisingly sophisticated microbial ecology studies in-flight without the availability of complex instrumentation. It also may be possible to enhance the performance of array hybridization with immobilized beacons.

In the work reported here we have already shown that our validated detector probes can be used to design useful molecular beacons, with our established methods. Efforts to find the optimal method of fragmenting the large RNAs should be continued in order to further enhance signals. In the future it would also be necessary to explore means of compensating for background from cellular autofluorescence by subtracting lysate background fluorescence calculated from off-wavelength excitation (at wavelengths at which flavin, etc. are the only active fluors, allowing their contribution at the beacon fluorophore wavelengths to be estimated). It also would be

appropriate to explore the use of infrared-wavelength fluors which are now freely commercially available from Licor. These fluors may possess significant advantages of background and turbidity penetration, especially near 1 micron wavelength).

Sample Preparation: Nucleic acid isolation and hybridization assays

While nucleic acid hybridization assays show promise for the identification of microorganisms, it is necessary to supply the assay with semi-purified DNA/RNA samples derived from the original environmental sample. In particular, it is essential to identify space-compatible methods of producing usable samples from blood, urine, and other crew-derived specimens, as well as water and bio-regenerative life-support system samples, which may contain microorganisms either essential or detrimental to their functioning. Liberation of nucleic acids from cells necessarily liberates comparatively large quantities of lipids, polysaccharides, membrane fragments, among other cellular constituents. Any of these materials can interfere non-specifically with the assay, and most of them are negatively charged, and so resemble nucleic acids in some important separations characteristics. The separation of DNA from, e.g. rRNA is especially problematic as they are similar in many physicochemical properties.

Most of the available technologies for nucleic acid isolation and sample preparations are not readily compatible with the spacecraft environment. These include phenol/chloroform extraction and CsCl-gradient banding. More appropriate will be adsorptive methods such as ion-pair reverse-phase and silica adsorption, and the ubiquitous anion-exchange adsorbents (e.g., 'Qiagen columns'). In order to enhance the chemical selectivity of RNA/DNA separation, discrimination against anionic polysaccharides, and primer removal from amplification (PCR/NASBA) mixtures, during the past funding period we have explored more specific techniques such as boronate affinity adsorption, immobilized-metal affinity chromatography (IMAC) and the use of compaction agents to enhance adsorption and, particularly, for selective precipitation. Compaction-agent-induced precipitation has proven widely useful for plasmid separation from RNA in alkaline lysis mixtures, and is now routinely used in multiple laboratories for preparation of sequencing templates and gene therapy vectors (the endotoxin level of the product is quite low). This technology is the subject of a pending U.S. patent application, and industrial licensing negotiations are well advanced. More recently we have extended the use of compaction precipitation (through the use of higher concentrations of more-potent precipitants such as hexammine cobalt) to precipitation and fractionation of rRNA from cell lysates. Stepwise precipitation yields an enriched mixture from which 5S or 16S rRNA can readily be isolated in substantially pure form. We routinely employ this method in preparing analytical standards for testing probes and beacons, and there is industrial interest in and demonstration of mRNA purification by this method. Most recently, we have initial, exciting results showing that the well-known immobilized-metal affinity chromatography technique is able to separate single- from double-stranded molecules, and RNA from DNA. This promising avenue should be further investigated.

Whole-Genome Expression measurement

With the advent of modern DNA technologies, the most direct and informative means of investigating the effects of environmental conditions is to study changes in fundamental levels of gene expression, using DNA probe hybridization assays. This approach is now taking a quantum leap in effectiveness, as the entire genomic sequences of numerous well-characterized model organisms are becoming available. The availability of whole-genome sequence information permits the design of hybridization probes for each and every gene expressed by the organism, so that in a single experiment one can simultaneously monitor the expression levels of all known genes. This approach would be ideal for rapidly determining the effects of the space environment on microorganisms and thereby understanding whether key properties such as growth rates, mutation rates or pathogenicity are likely to be affected. If a hybridization array instrument were developed for microbial monitoring it would also be useable for in-flight analysis of global gene expression studies. This would require mRNA purification in-flight, which could be accomplished using the methods that have been developed during the last three-year funding period.

APPENDIX A: PROJECT DATA

The following appendix material consists of tables and figures, which contain representative data from the four major project areas; probe design, probe validation, assay design and sample preparation. This brief text will summarize what is shown in the various figures. Further detail is available in the figure captions.

The first group of figures and tables relate to probe design. This set begins with n illustration of 16S rRNA showing the major secondary structure elements that might make probe access difficult. This is followed by a second figure, which shows the locations of successful probes designed by others and us. These figures are followed by Table 1, which provides the source code for the program OLIGOSCAN that was extensively used in designing probe.

The second group begins with Table 2, which lists the probes that were successfully validated for use in a water assay system. The subsequent figures illustrate the type of testing that was used to establish the utility of the probes shown in Figure 2. Figures 3-5 primarily follow probes E1 and E2, which are *E. coli* specific. It is shown in these figures that these probes not only recognize *E. coli* as intended but also do not recognize either distant organisms or very closely related enteric organisms.

The next group of figures, 7-16 illustrates the two assay formats that were investigated. These were a DNA hybridization array assay in a sandwich format that was tested in collaboration with Genometrix Inc. and a solution format using molecular beacons. Illustrative array hybridization results are shown in Figure 9. The molecular beacon approach is described in schematic form in Figure 10. Figure 11 shows the fluorescent intensity and anisotropy kinetics for beacon hybridization to complementary deoxyoligonucleotide. Figure 12 illustrates the thermal denaturation profiles of beacon and beacon/target hybrid. Figure 13 shows the titration and saturation of beacon signal as a function of 5S rRNA concentration, and the lower panel the calibration curve for 5S rRNA detection using the beacon. Figure 14 illustrates the efficient combined cell-permeabilization/beacon hybridization assay, and the specificity of this assay for target organisms. Figure 15 shows the specific detection of *V. proteolyticus* against an increasing background of *E. coli* cells, and the detection of *E. coli* expressing *V. proteolyticus* 5S rRNA against a background of unlabeled cells. Although the beacon technology is very promising it is difficult for the beacon to access its target in the whole 16S rRNA. We therefore investigated methods, Figure 16, to fragment the target RNA without destroying the actual target sequence.

The final aspect of the project was the development of spacecraft compatible methods for processing samples. Figures 17-35 relate to the use of compaction agents to enhance adsorptive and especially precipitation-based nucleic acid isolation for sample preparation. Figures 36-42 describe our work on the use of immobilized-metal affinity chromatography (IMAC) for isolation of nucleic acids. Figure 17 illustrates the structures of common compaction agents. Figure 18 shows a seminal early finding - at low ionic

strength, DNA can be selectively precipitated away from RNA. Figure 19 shows that extremely low ionic strength is not required, if slightly higher levels of spermidine are used, and Figures 20 and 21 illustrate the practical separations that can be achieved using compaction precipitation. Figures 22-24 show the enhancement of adsorptive separation (anion-exchange) possible by compaction of nucleic acid to reduce steric hindrance. Tables 25, 26 and 27, and Figures 28 and 29, summarize the results for DNA adsorption with various compaction agents, and show the Hill plots used to analyze adsorption affinity and heterogeneity. Figure 30 shows light-scattering-monitored compaction of plasmid, linear DNA, and total RNA by several compaction agents. Figures 32-35 show RNA separations achievable with compaction precipitation.

Figure 36 begins the section on IMAC metal-affinity purification of nucleic acids, showing the adsorption isotherms for baker's yeast RNA on IDA-sepharose charged with various metals. The results shown here illustrate the superiority of copper and nickel in this application. Figure 37 shows adsorption isotherms for DNA homopolymers on nickel-charged IDA-sepharose, showing the preferential binding of purines, and absence of affinity for double-stranded DNA. Figures 38-40 show practical RNA and DNA separations achievable with IMAC. Figure 41 illustrates the recent detection of a mismatch in a 20-mer duplex using IMAC - this result may presage the use of IMAC HPLC or CE in hybridization assays and/or SNP detection.

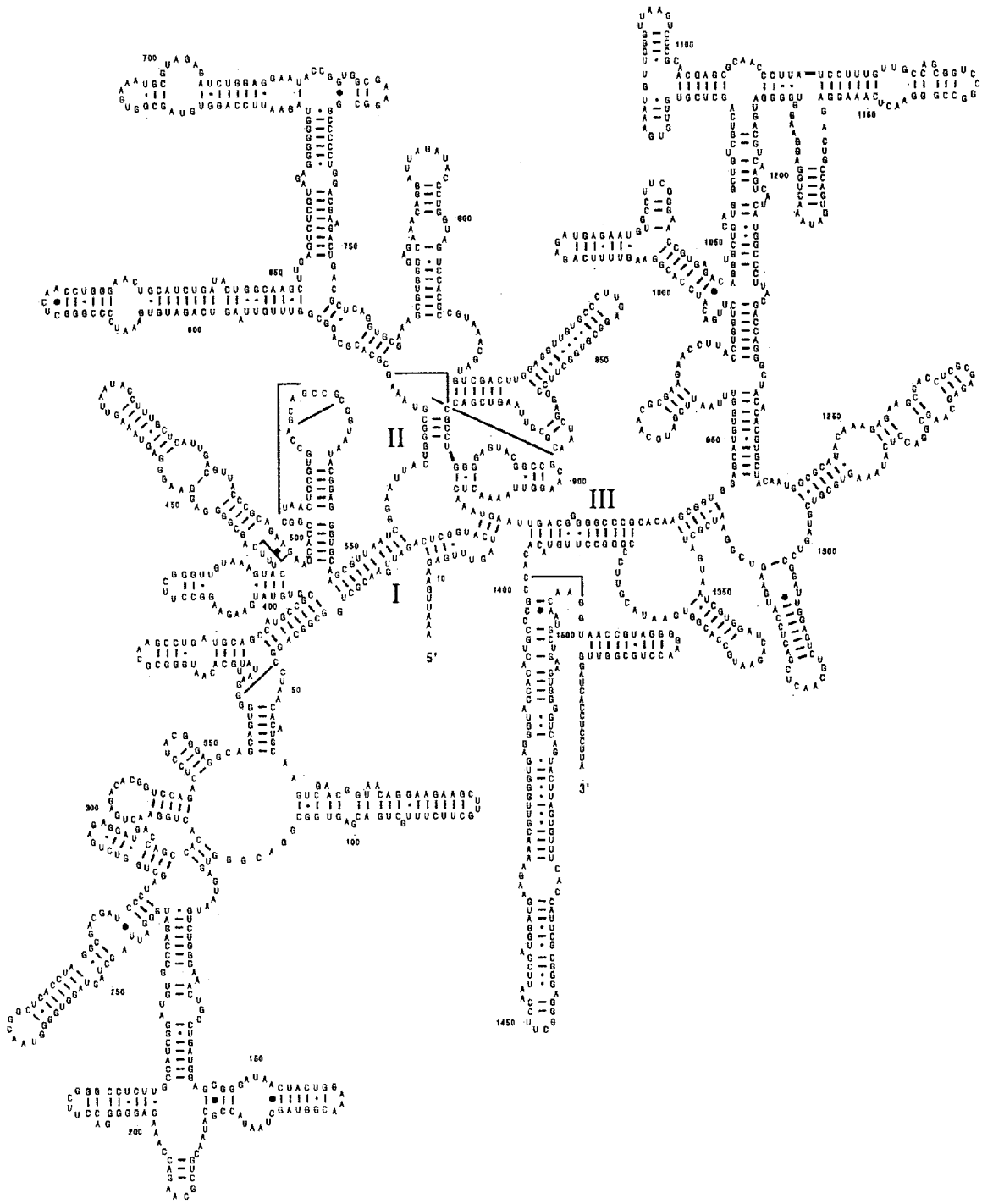


Figure 1. Secondary structure of *Escherichia coli* 16S ribosomal RNA. This figure was modified from that available at www.pundit.icmb.utexas.edu/RNA/.

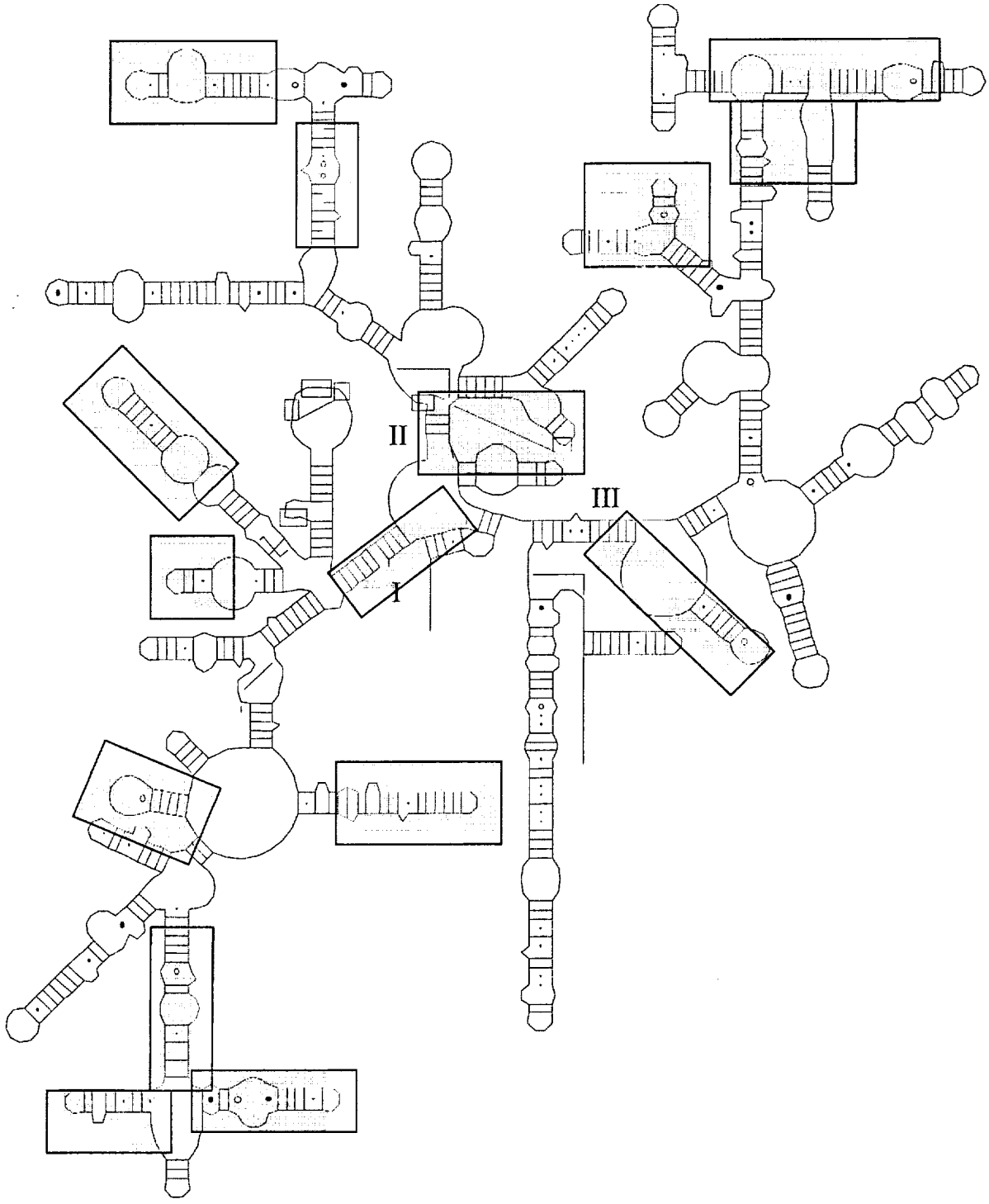


Figure 2. General regions on 16S rRNA that have been targeted successfully using genus and species specific oligonucleotide probes (figure compiled using data from http://www.cme.msu.edu/OPD/).

Table 1: **SOURCE CODE "OLIGOSCAN"**

```
#!/usr/local/bin/perl
        print "Input the fileto be scanned for unique oligos\:";
        $input_file = <STDIN> ;
        chomp $inputfile ;
        open (FILE,"$input_file");
         print "Enter the name of the output file:\n";
     $out = <STDIN>;
     chomp $out;
         `rm $out` if -e "$out";
        open (OUT,">>$out");
        $i=1;
        foreach $line (<FILE>)
        {       chop $line;
                if ($line=~ /\>/)
                {
                        $line=~ s/\s+/\_/ig;
        $locus {"SEQ$i"} ="$line";
        push(@fullseq,"\:SEQ$i\>");
                        $i++;
                        } else {
                        $line =~ s/\s\n//ig ;
                        push(@fullseq,"$line");
                }
                }
$fullseq="@fullseq";
$fullseq =~ s/\s//ig;
#print "$fullseq\n\n\n\n";
                close (FILE);
        print "Please input the length of the oligos:\n";
        $window= <STDIN>;
        chomp $window;
        print "What is maximum number of mismatches allowed:\n";
        $match = <STDIN>;
        chomp $match;
        print `clear`;
        print "                There is nothing that you cannot solve with adequate doses of caffeine      \n";
        $begin_time= (times) [0];
        $i=1;
        {
        @seq = split(/:/, $fullseq);
        }
        $length = @seq;
        for ($lines=1;$lines<$length;$lines++)
        {
                $string="$seq[$lines]";
                ($header,$string1) = split (/\>/, $string);
                print "\n$header\t";
                print OUT "\n$header\t";
                print $locus{"SEQ$lines"};
                print OUT $locus{"SEQ$lines"};
                chomp $string1;
                $length_string = length($string1);
                print "\tNumber of nucleotides:$length_string\n";
```

```
                print OUT "\tNumber of nucleotides:$length_string\n";
                @stringx="";
                $lengthtwo=0;
                &compare;
        }
        $end_time= (times) [0];
    $CPUtime = $end_time - $begin_time;
    print "\n\n\tREPORT\n\nThe database scanned was: \t$input_file";
        print OUT "\n\n\tREPORT\n\nThe database scanned was: \t$input_file";
        print "The length of the Oligos selected: \t$window\n";
                print OUT "The length of the Oligos selected: \t$window\n";
        print "The maximum no. of mismatches allowed: \t$match\n";
                print OUT "The maximum no. of mismatches allowed: \t$match\n";
    print "The program took $CPUtime CPU seconds.\n";
        print OUT "The program took $CPUtime CPU seconds.\n";
sub compare {
                for ($anotherline=0;$anotherline<$length;$anotherline++)
                        {
                        $string2a="$seq[$anotherline]";
                        ($header2,$string2)= split (/\>/,$string2a);
chomp $header2;
                        chomp $string2;
                        if ($string1 eq  $string2)
                                {
                                if($header ne $header2)
                                {
                                print "\*NOTE:$header is identical to another sequence in the database
$header2\*\n";
                                print OUT "\*NOTE:$header is identical to another sequence in the database
$header2\*\n";
                                        }
        #$anotherline=$length-1;
                        }
                                else
                                {
                                chomp $string2;
                                $lengthtwo=$lengthtwo+length($string2);
                                push(@stringx,$string2);
                                }
                        }
                        chomp @stringx;
                        $limit=($lengthtwo-$window);
                        &compsubstring;
                }
sub compsubstring
        {
        for($start=0;$start<((length($string1)-$window)+1);)
                {
                $supercounter=0;
                $super=0;
                for($starttwo=0;$starttwo<(($lengthtwo-$window)+1);$starttwo++)
                        {
                        $substring1= substr($string1,$start,$window);
                        $stringx="@stringx";
                        $stringx=~ s/\s//ig;
                        $substring2= substr($stringx,$starttwo,$window);
```

```
                        &score;
                        if ($supercounter==23000000)
                                {
                                last;
                                }
                                elsif ($supercounter==$limit)
                                        {
                                        $supercounter=0;
                                        $super=$super+1;

                                        }

                        else
                                {
                                $super=$super+0;
                                }
                                }
                        if ($super != 0)
                                {
                                print "$substring1\tat position $start \n";
                                print OUT "$substring1\tat position $start \n";
                                }
                        $start=$start+1;
                }
                }
sub score
        {
        @array_substring1= split (//, $substring1);
        @array_substring2= split (//, $substring2);
        $length_array=@array_substring1;
        $counter=0;

        for($count=0;$count<$length_array;$count++)
                {
                $reversecount=$length_array-$count;
        if (($array_substring1[$count]) eq ($array_substring2[$count]) || ($array_substring1[$reversecount]) eq
($array_substring2[$count]))
                        {
                        $counter=$counter+1;
                        }
                else
                        {
                        $counter=$counter+0;
                        }
                }
                if ($counter > $match)
                        {
                        $supercounter == 23000000;
                        last;
                        }
                        else
                        {
                        $supercounter = $supercounter+1;
                        }
                }
```

PROBE	SEQUENCE (5'-3')	ORGANISMS HIT	BASES IN *E. coli*
16S-R	AGAAGGAGGTGATCCACGCA	All bacteria	1522-1541
Pan 339	CTGCCTCCCGTAGGAG	All bacteria	339-354
Pan 780	AGGGTATCTAATCCTGTTT	All bacteria	780-798
Acineto 414	GCTTTACAACC(A/C)(A/T)AAGGCCT	*Acinetobacter* sp	414-433
A6	TAGTGAACACTGGAA	*Acinetobacter*	165-179
Acineto 437	AGCCTCCTCCTCGCTTAAA	*Acinetobacter*	437-455
Burk 803	CAT(C/G)GTTTAGGGCGTGGAC	*Burkholderia cepacia*	803-821
Burkchap	TTCCGGTACCGTCATCCC	*Burkholderia cepacia*	444-461
A7	GACTCTCCGCCCTCACG	*Burkholderia cepacia*	1006-1022
A3	TAGCCGCAGCTCG	*Vibrio proteolyticus*	*99-111*
A4	TCAATTATCTACGTAAT	*Vibrio proteolyticus*	100-116
A5	TGCGAGAGTGAAACACC	*Vibrio proteolyticus*	1464-1480
E1	AGCAAAGGTATTAACTTTACTCCCT	*Escherichia coli*	452-476
E2	TTCCCGAAGGCACATTCT	*Escherichia coli*	1019-1036
E3	TTCCCGAAGGCACCAATC	gram negatives	1019-1036
Ent 2	TAGTTATCCCCCTCCATCAGGCAGT	enterics	131-157

Table 2. Tabulation of validated probes.

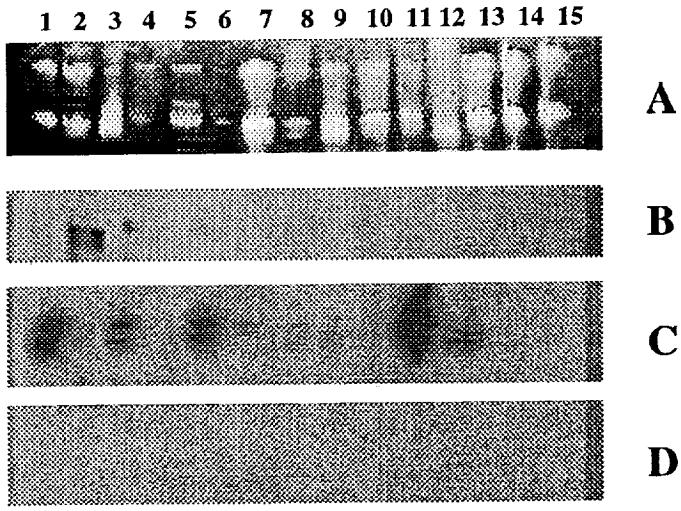


Figure 3. Panel A: ribosomal RNA preparations from the following organisms: 1. *Escherichia coli*, 2. *Vibrio proteolyticus*, 3. *Enterobacter aerogenes*, 4. *Acinetobacter* sp., 5. *Proteus vulgaris*, 6. *Proteus mirabilis*, 7. *Pseudomonas aeruginosa*, 8. *Pseudomonas putida*, 9. *Burkholderia cepacia*, 10. *Serratia marcescens*, 11. *Klebsiella pneumoniae*, 12. *Staphylococcus aureus*, 13. *Staphylococcus xylosus*, 14. *Enterococcus faecalis*, and 15. *Bacillus subtilis*. Panel B: A3, a *Vibrio proteolyticus*-specific probe, Panel C: Ent 3, an enteric-specific probe, Panel D: A13, a *Staphylococcus aureus* specific probe, which showed no hybridization with its target RNA.

Validation of Potential *E. coli* Probes

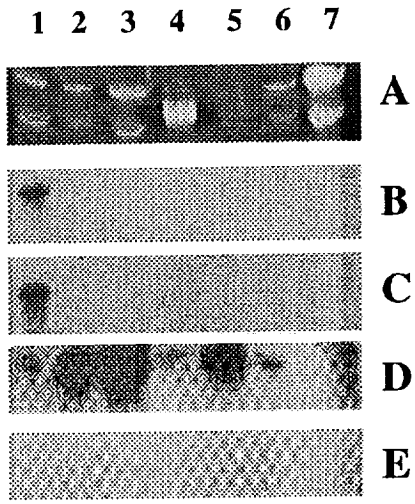


Figure 4. Organism specific probes were tested using hybridization with ^{32}P-labeled oligonucleotides and viewed by autoradiography. Panel A shows the large ribosomal RNAs (23S and 16S) ran on a 8M urea polyacrylamide gel (3.5%). Lane 1: *E. coli* rRNA, lane 2: *Vibrio proteolyticus* rRNA, lane 3: *Enterobacter aerogenes* rRNA, lane 4: *Pseudomonas aeruginosa* rRNA, lane 5: *Pseudomonas putida* rRNA, lane 6: *Burkholderia cepacia* rRNA, and lane 7: *Staphylococcus xylosus* rRNA. Panel B-E show the autoradiography results obtained for several suspected *E. coli* and *S. aureus* specific probes. B: probe E1 (targets *E. coli*), C: probe E2 (targets *E. coli*), D: probe E3 (targets *E. coli*), and E: probe A13 (targets *S. aureus*). As can be seen from the autoradiographs, probes E1 and E2 successfully target *E. coli* 16S rRNA and show no signal with the other organisms. The E3 probe is not specific for *E. coli* alone, but targets only gram negative organisms and not gram positive organisms, and hence will be used as a gram-negative detector. The *S. aureus* probe, A13, did not hybridize to any of the rRNAs, including its target. Further *Staphylococcus* probe design is presently being done.

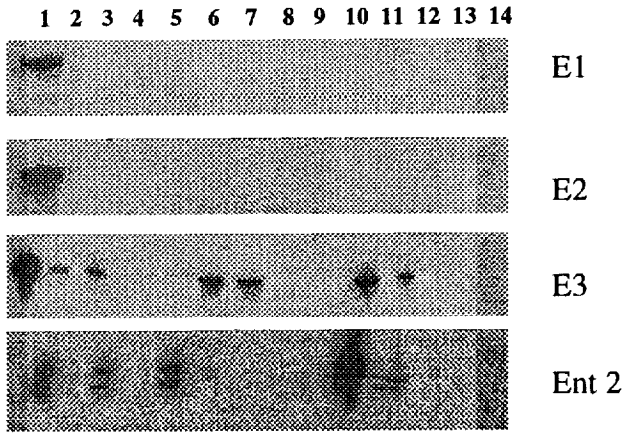


Figure 5. Specificity of *E. coli* probes (E1, E2, E3) and the enteric probe Ent 2. 1. *E. coli*, 2. *Vibrio proteolyticus*, 3. *Enterobacter aerogenes*, 4. *Acinetobacter* sp., 5. *Proteus vulgaris*, 6. *Proteus mirabilis*, 7. *Pseudomonas aeruginosa*, 8. *Pseudomonas putida*, 9. *Burkholderia cepacia*, 10. *Serratia marcescens*, 11. *Klebsiella pneumoniae*, 12. *Staphylococcus xylosus*, 13. *Enterococcus faecalis*, 14. *Bacillus subtilis*. Ent 2 hits all enteric organisms assayed, while probes E1 and E2 taget only E. coli 16S RNA. Probe E3 hits gram negatives and no gram positive organisms. The gram negative organisms not hit by this probe in this figure (lanes 4, 5, 8 and 9) are due to low concentrations of target RNA and are not a reflection of probe specificity.

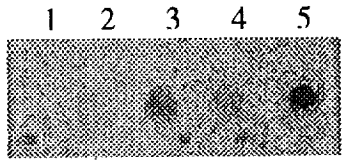


Figure 6. Autoradiograph showing results obtained after dot-blot hybridization with A7, a probe specific for *Burkholderia cepacia*. Each lane contains purified 16S rRNA from 1. *E. coli*, 2. *Vibrio proteolyticus*, 3. *Pseudomonas aeruginosa*, 4. *Pseudomonas putida*, and 5. *Burkholderia cepacia*.

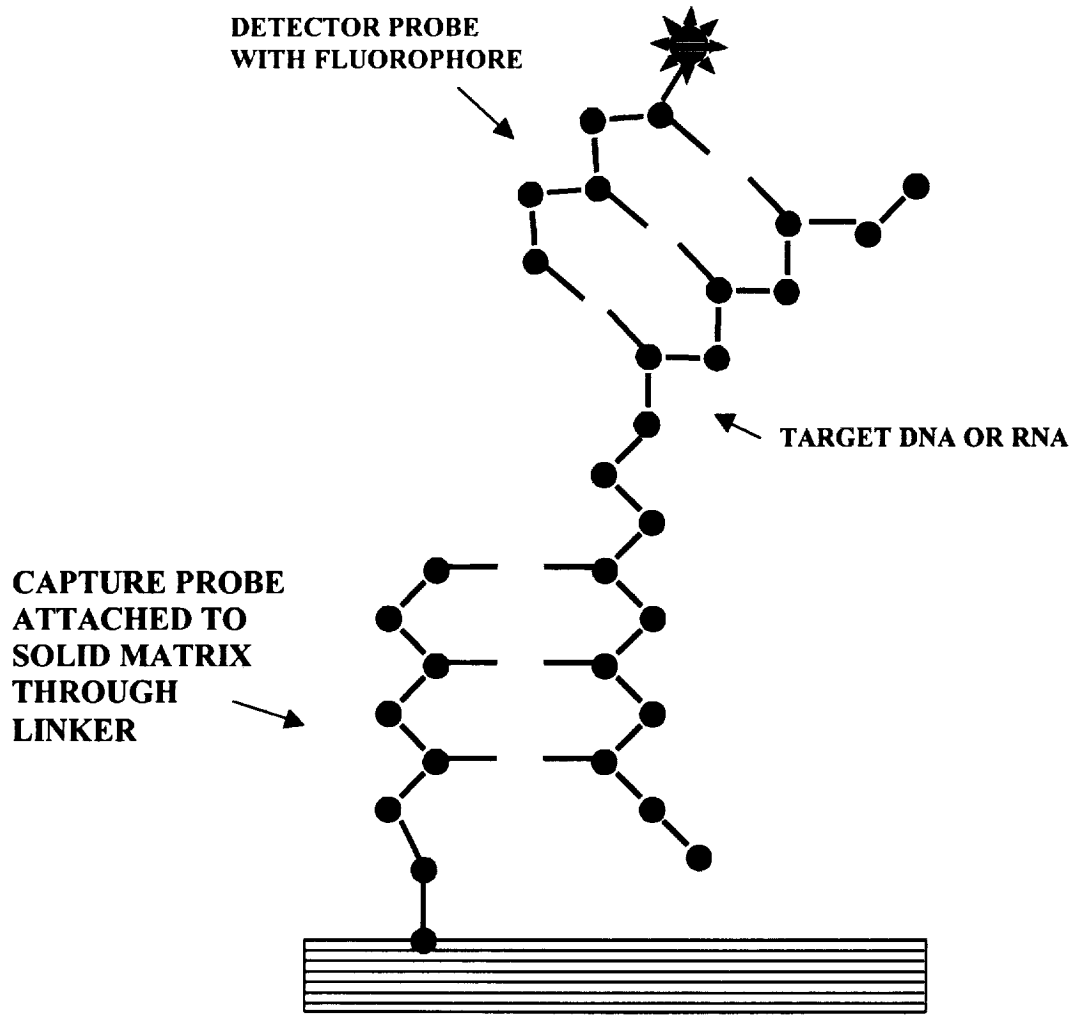


Figure 7. Cartoon depicting hybridization using a "sandwich" assay. The target is hybridized with the labeled detector probe first, and then added directly onto the solid matrix, where further hybridization to the capture probe can occur. After washing, only target that has the detector probe bound will fluoresce.

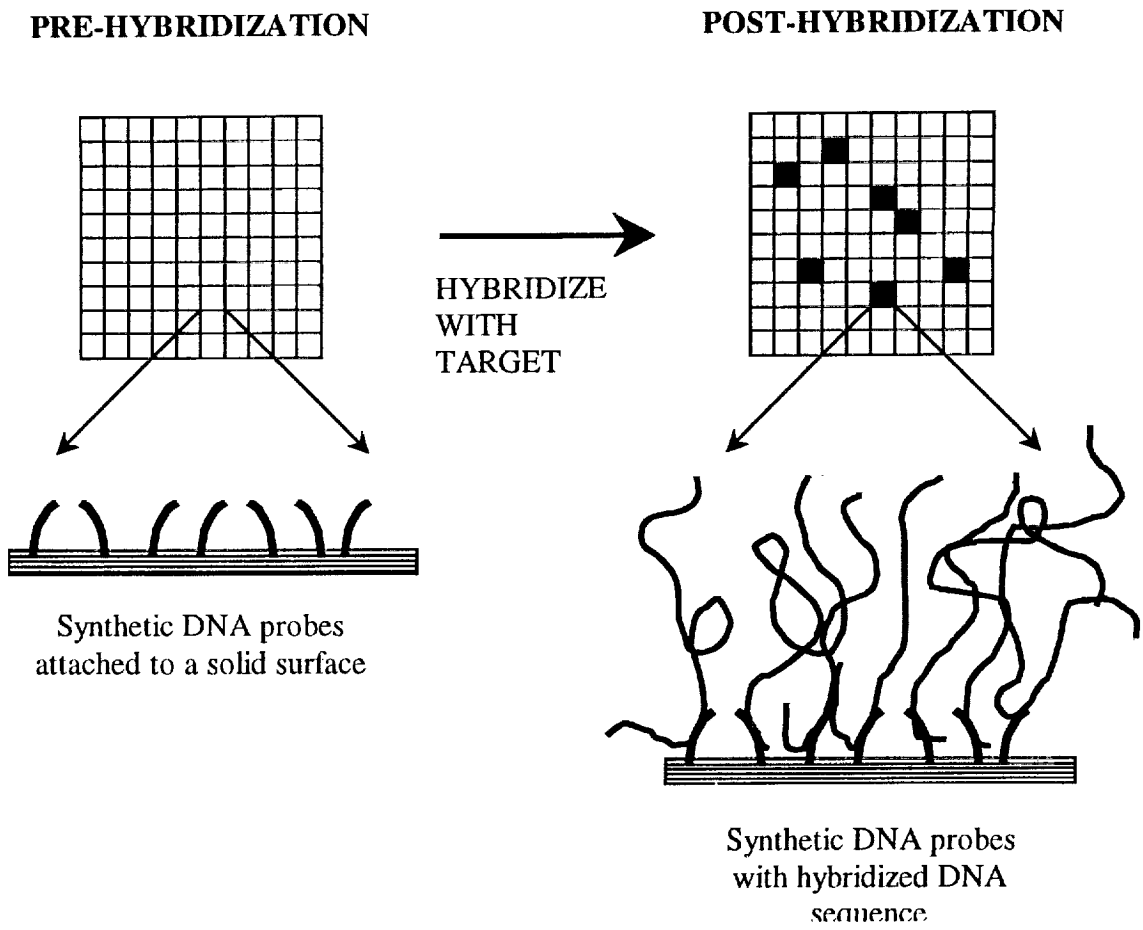


Figure 8. Hybridization assay on a DNA chip. A two-dimensional pattern is generated when target DNA (or RNA) binds to the capture probes attached to the solid matrix of the chip, forming a detectable hybrid. This pattern can be quickly analyzed by an instrument such as a fluorescence plate reader, to determine which organisms are present, and in what amount.

Genometrix DNA chip

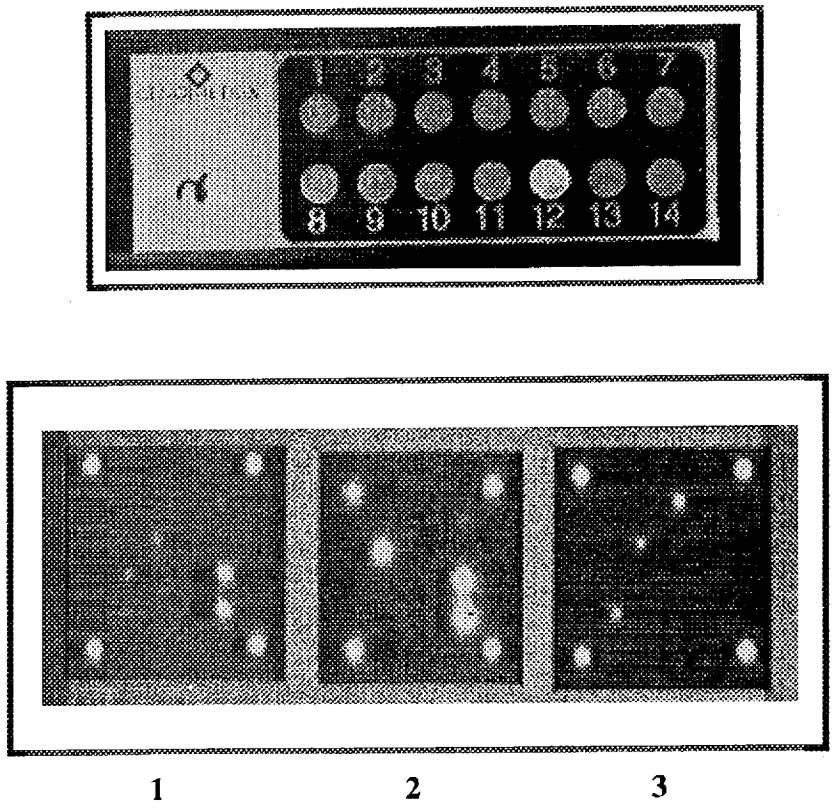


Figure 9. DNA chip hybridization results after detector probe is excited and the chip is viewed with a fluorescent microscope. The chip was hybridized with samples from 1. *Burkholderia cepacia,* 2. *Ralstonia pickettii*, and 3. main effluent tank.

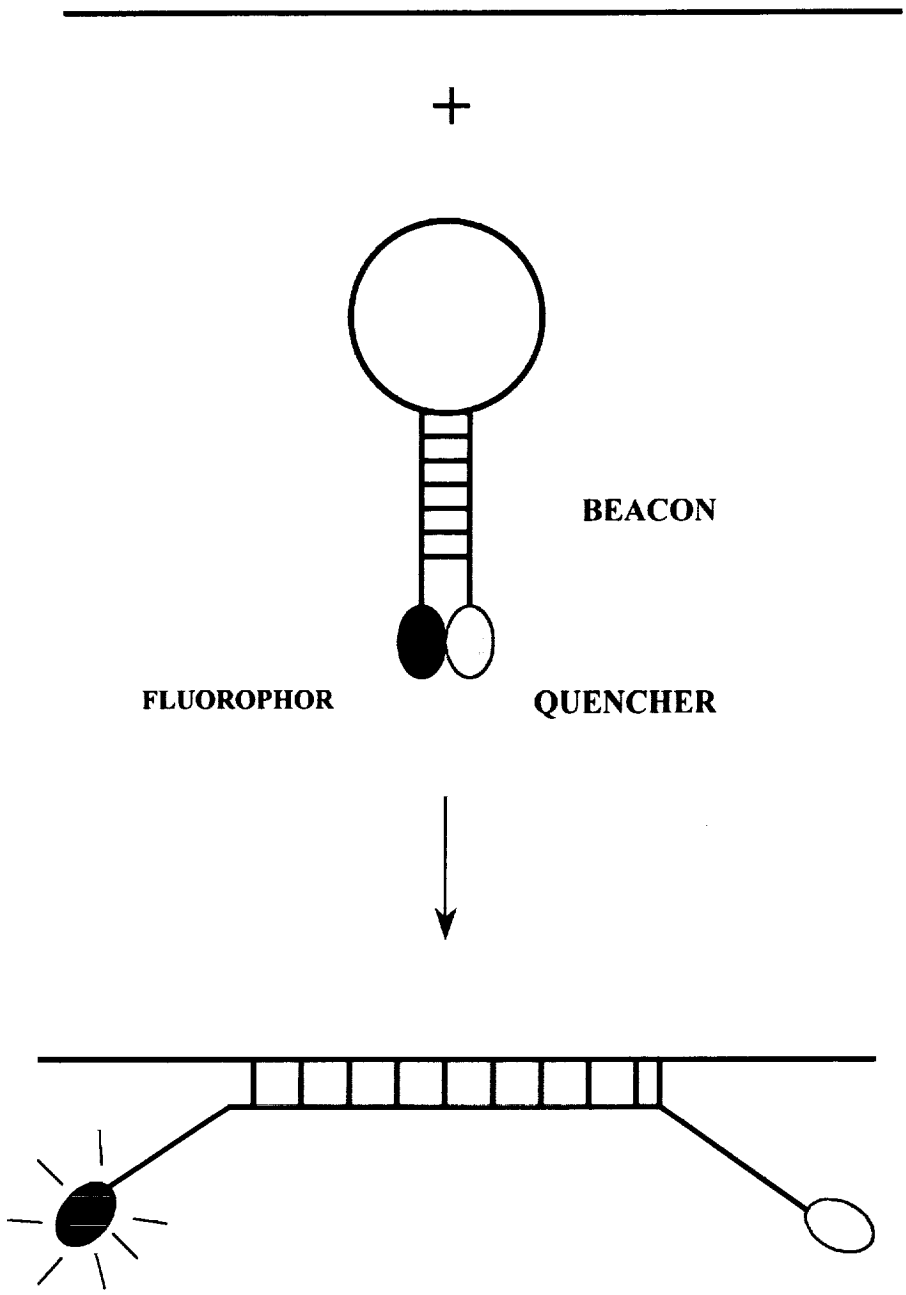


Figure 10. Basis of the molecular beacon technology. The beacon is a small DNA oligomer which initially forms a stem-loop structure, such that an attached the fluorophore and quencher are in close proximity. As a result, fluorescence is quenched due to FRET. However, the single strand loop of the beacon DNA , contains sequences complementary to a specific DNA or RNA target (shown in red).. In the presence of this target, hybridization occurs, disrupting the stem-loop conformation. The fluorophore and the quencher are forced apart, and fluorescence is restored and can be detected.

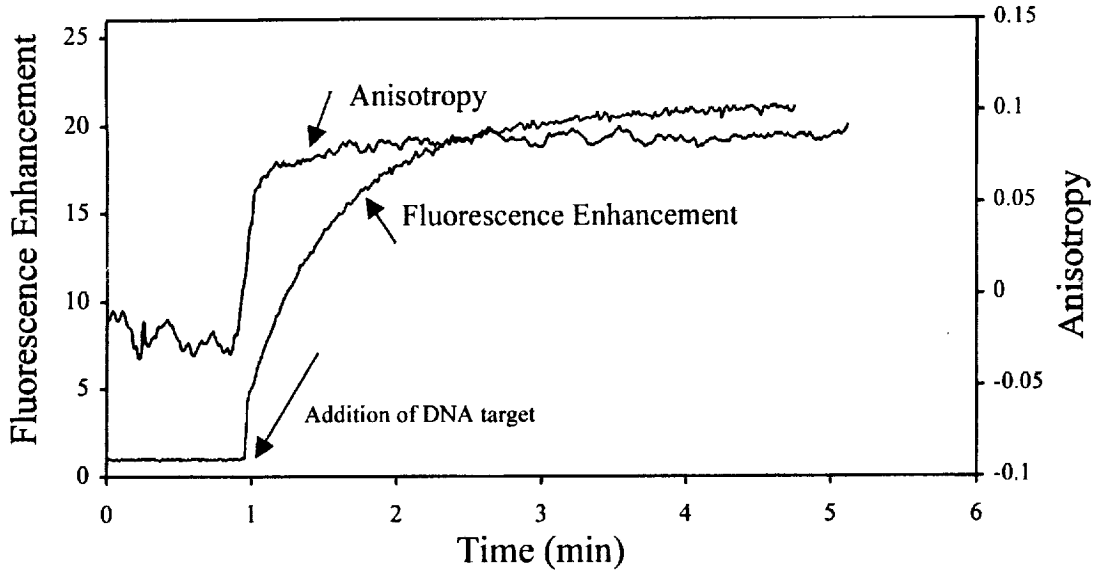


Figure 11
Association of the molecular beacon (10 nM) to a 12-fold molar excess of the complementary deoxyoligonucleotide in 600 µl hybridization buffer, at 25°C. The fluorescence of the solution of the molecular beacon was monitored for 4 minutes before the addition of the target. The enhancement ratio was calculated as described in the text. Final intensity was 7.4×10^5 cps (emission filter). Anisotropy changes were recorded during hybridization using T-formal optics of the fluorometer .

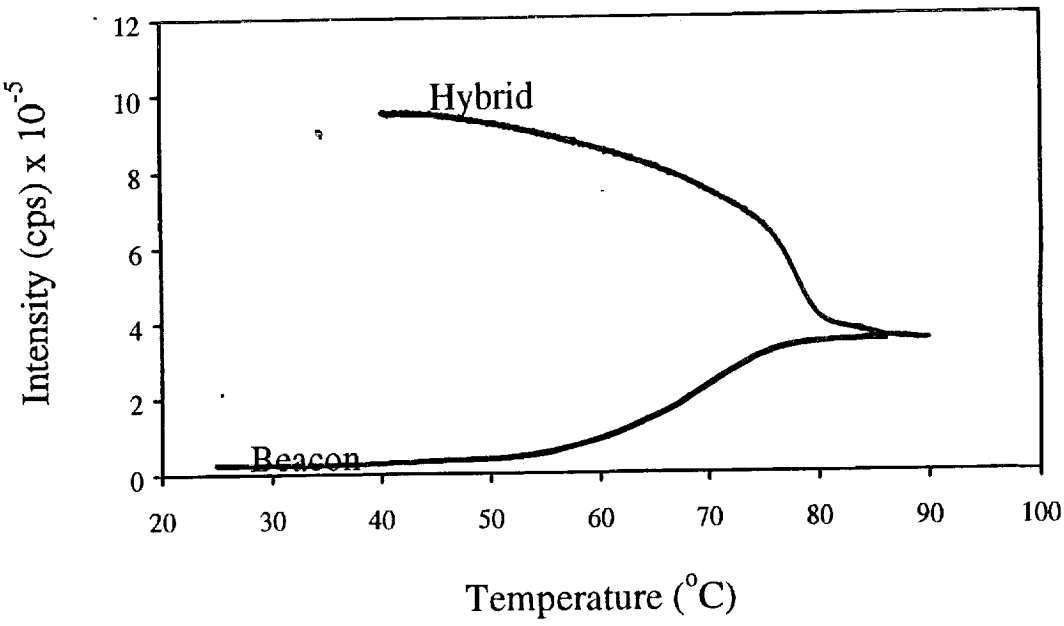


Figure 12

Thermal denaturation profiles of the beacon (50 nM) and the beacon-oligonucleotide hybrid. As the temperature increases the quenched fluorescence is restored because denaturation of the hairpin structure separates the quencher from the fluorophore moiety. Increasing the temperature dissociates the hybrid; the decrease in the fluorescence can be attributed to residual interactions between the quencher and fluorophore in the denatured beacon.

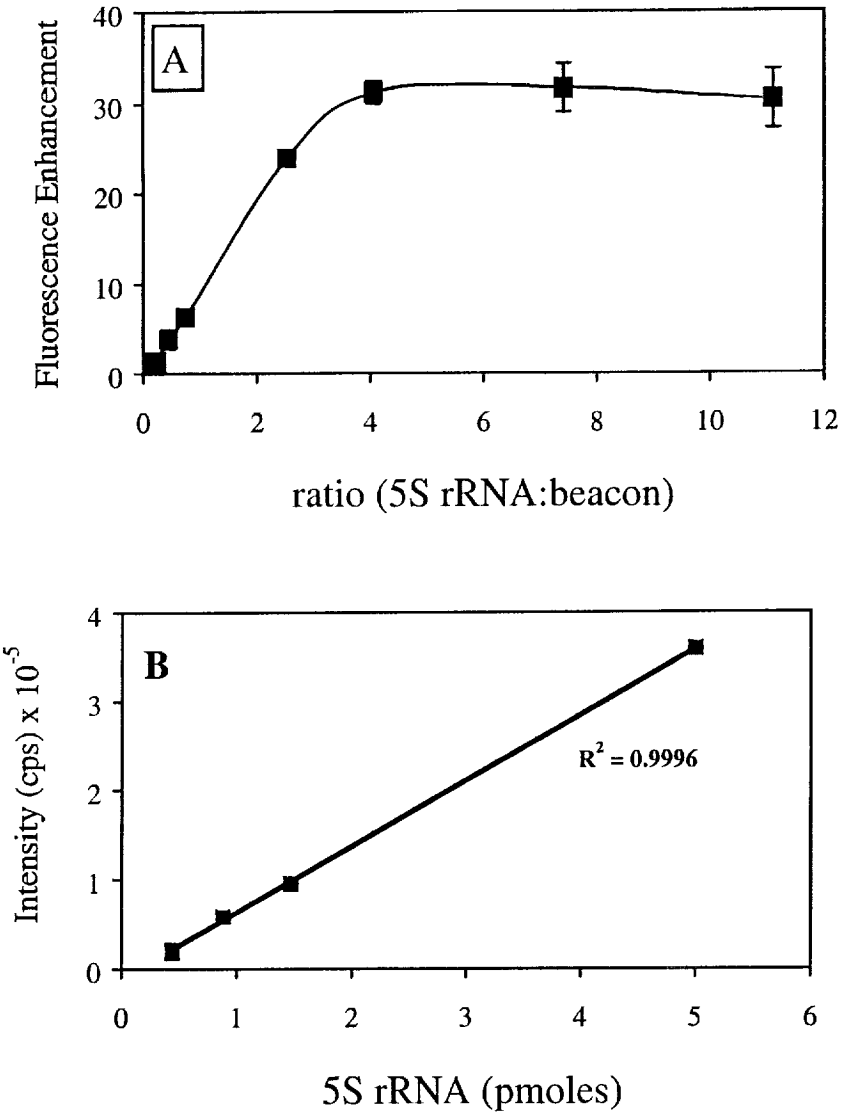


Figure 13

A. Influence of the molar ratio of probe to target on the fluorescence enhancement of the hybridization of the FAM molecular beacon (3.2 nM) to *V. proteolyticus* total rRNA (0-15.0 μg). The samples were run in duplicate. Fluorescence values were recorded after an overnight incubation at 37°C. B. Standard curve for *V. proteolyticus* 5S rRNA measured with FAM molecular beacon (3.2 nM). Linear detection of the target with molecular beacons is obtained in the picomolar range. Samples were run in duplicate.

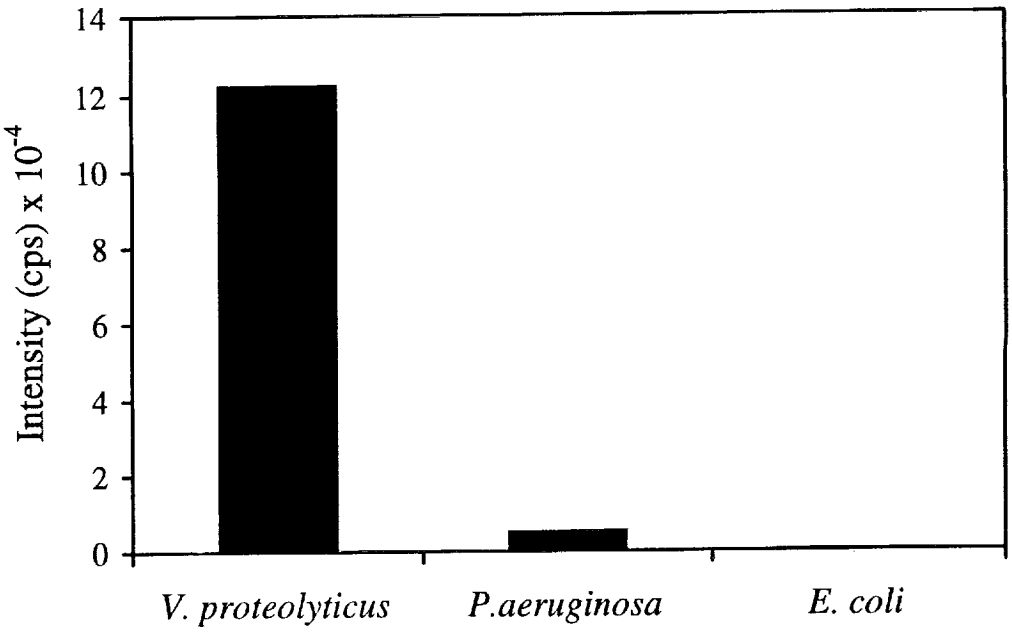


Figure 14

Combined Cell permeabilization/hybridization assay. 5 µl solution of *V. proteolyticus*-specific FAM molecular beacon (1.1 µM) was added to 100 µl sample containing cell lysate supernatants produced by heating from different bacterial strains. Hybridization was carried out as described in Materials & Methods and fluorescence intensity was recorded after overnight incubation at 37°C and dilution of the samples with 0.5 ml hybridization buffer. The fluorescence intensity of unhybridized molecular beacon (in buffer solution with no cell lysate) was $2.9 * 10^4$ cps and was subtracted from the signal for each sample.

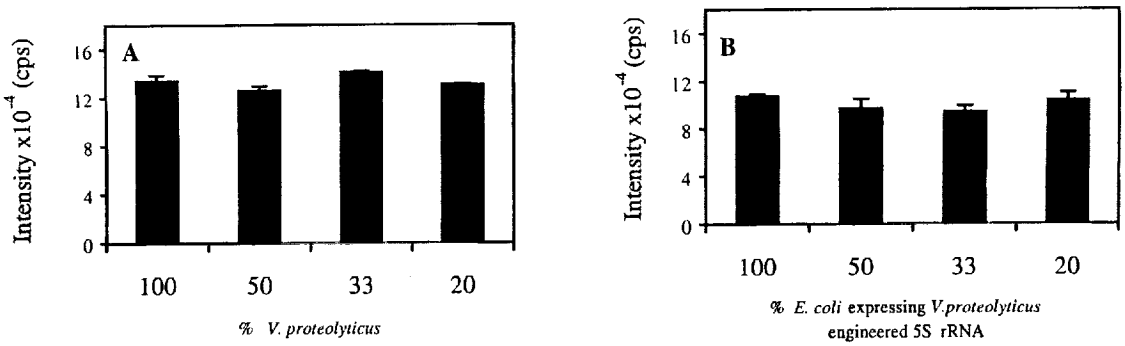


Figure 15

A. Detection of *V. proteolyticus* cells in the presence of excess *E. coli* cells. Cell pellets (approximately 10^{10} cells) were resuspended in 0.2 ml hybridization buffer, heat-treated (95 °C, 5 min) and centrifuged (10, 000 rpm, 10 min). The supernatant was diluted 1:2 with hybridization buffer and aliquots of the solutions were hybridized to FAM molecular beacon (25 pmoles in 160 µl assay volume). 10 µl of *V. proteolyticus* cell lysate supernatant was hybridized in the presence of variable amounts of *E. coli* cell lysates supernatant (10 – 40 µl). Fluorescence intensity was recorded after an overnight incubation at 37°C.

B. Detection of labeled *E. coli* cells in the presence of excess unlabeled *E. coli* cells. Lysate supernatant of *E. coli* JM109, expressing engineered *V. proteolyticus* 5S rRNA was hybridized to FAM molecular beacon (the procedure followed was the same as in A) in the presence of variable amounts of *E. coli* cell lysate supernatant. For both cases the hybridization to the target RNA was unaffected by the presence of non-target species.

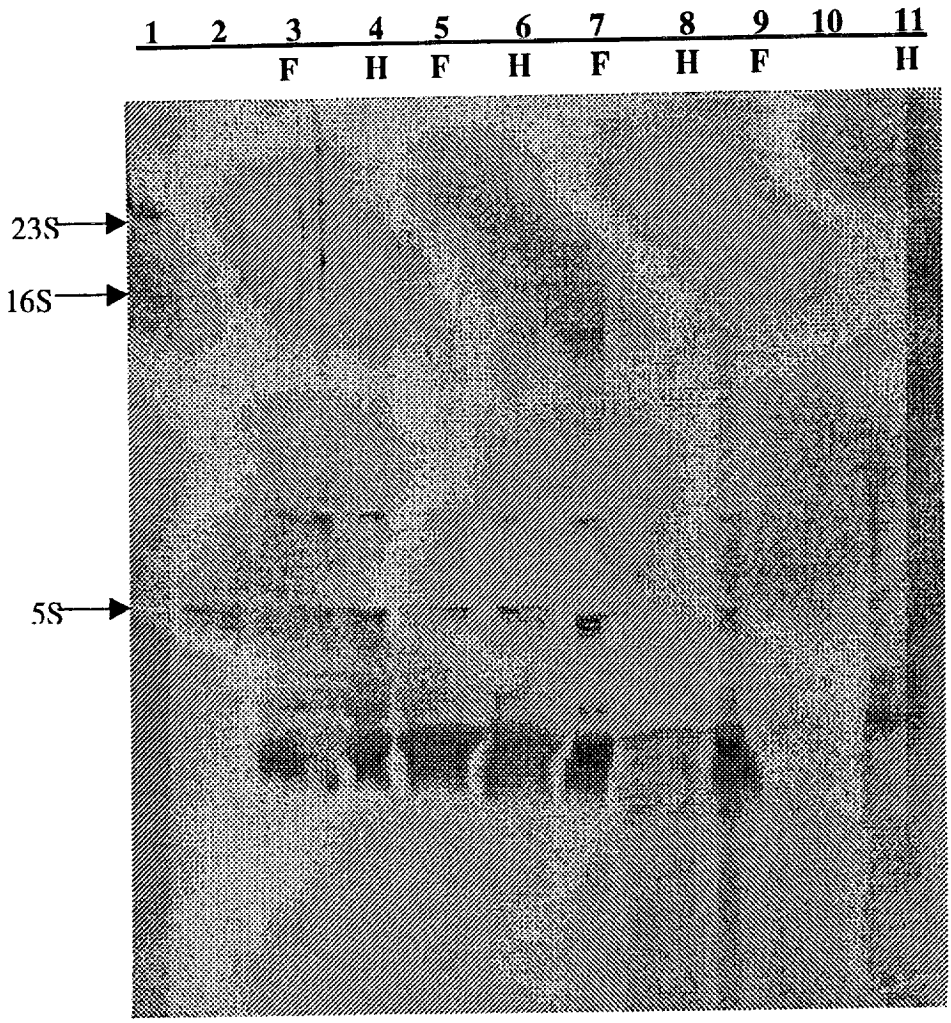
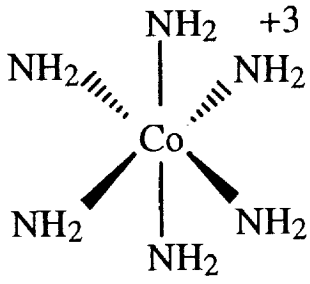
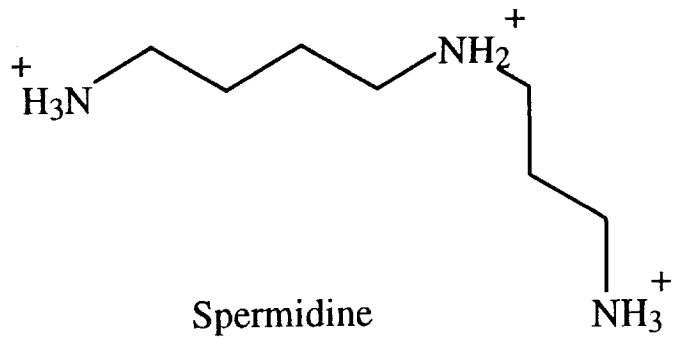
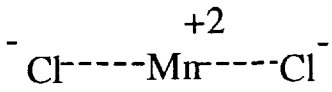


Figure 16. Fragmentation of total RNA will likely be necessary for solution hybridization with 16S rRNA. This will provide accessible target regions for the molecular beacon probes. Fragmentation of rRNA into ~100 base fragments was achieved using two different buffers. Total *E. coli* RNA was incubated with buffer F (40 mM tris acetate (pH 8.1), 100 mM potassium acetate, 30 mM Mg acetate) or buffer H (10 mM tris (pH 8), 50 mM KCl, 10 mM $MgCl_2$) at 95°C for different time frames. RNA samples were then separated in an 8M urea polyacrylamide stacking gel (3.5%/10%). 1: total *E. coli* RNA, 2: 5S rRNA (marker), 3-4:1 min, 5-6: 2 min, 7-8: 10 min, 9,11: 30 min. Lane 10 was left blank due to leaking from lane 9 while loading samples onto the gel.



Hexammine Cobalt





Manganese Chloride

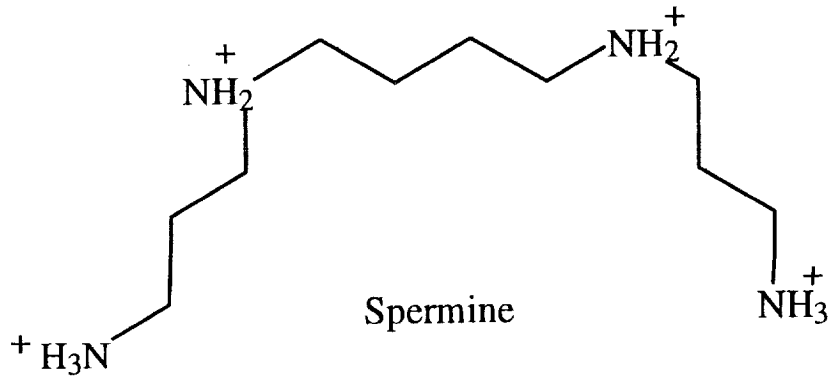


Figure 17: Structures of common compaction agents.

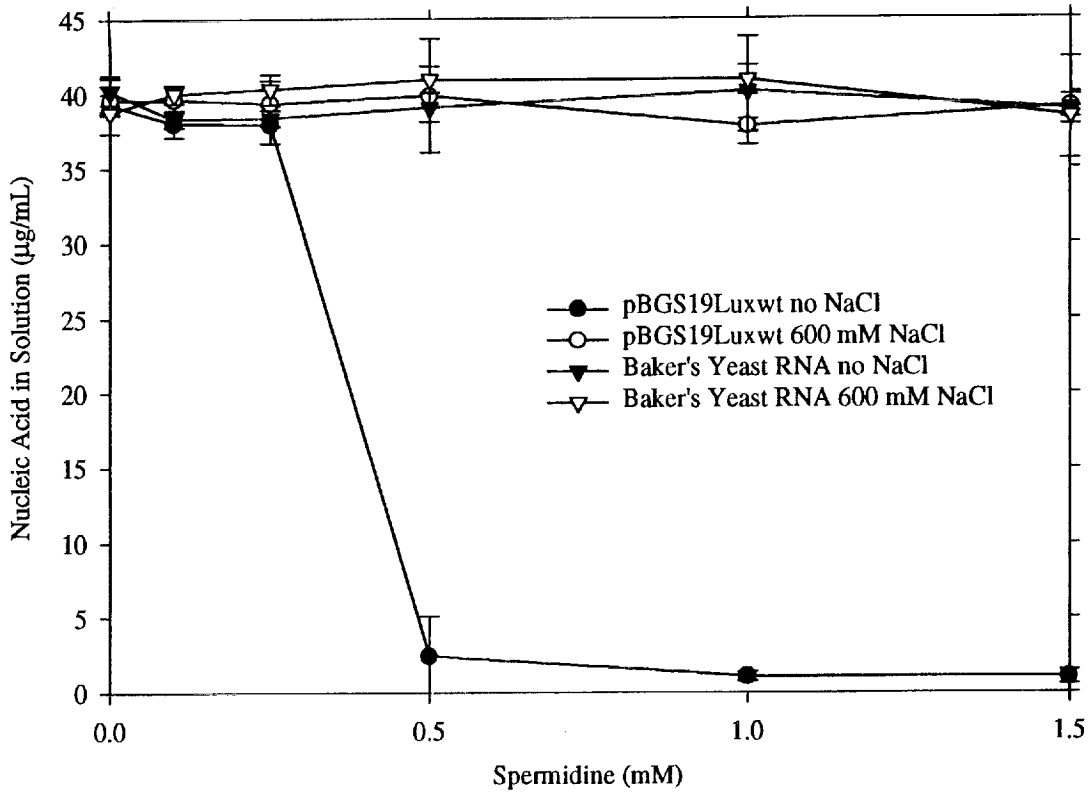


Figure 18:

Precipitation by spermidine of 40 µg/mL pBGS19luxwt or Baker's yeast RNA in 10 mM Tris buffer at pH 8.0 with and without 600 mM NaCl. Error bars are +/- one standard deviation.

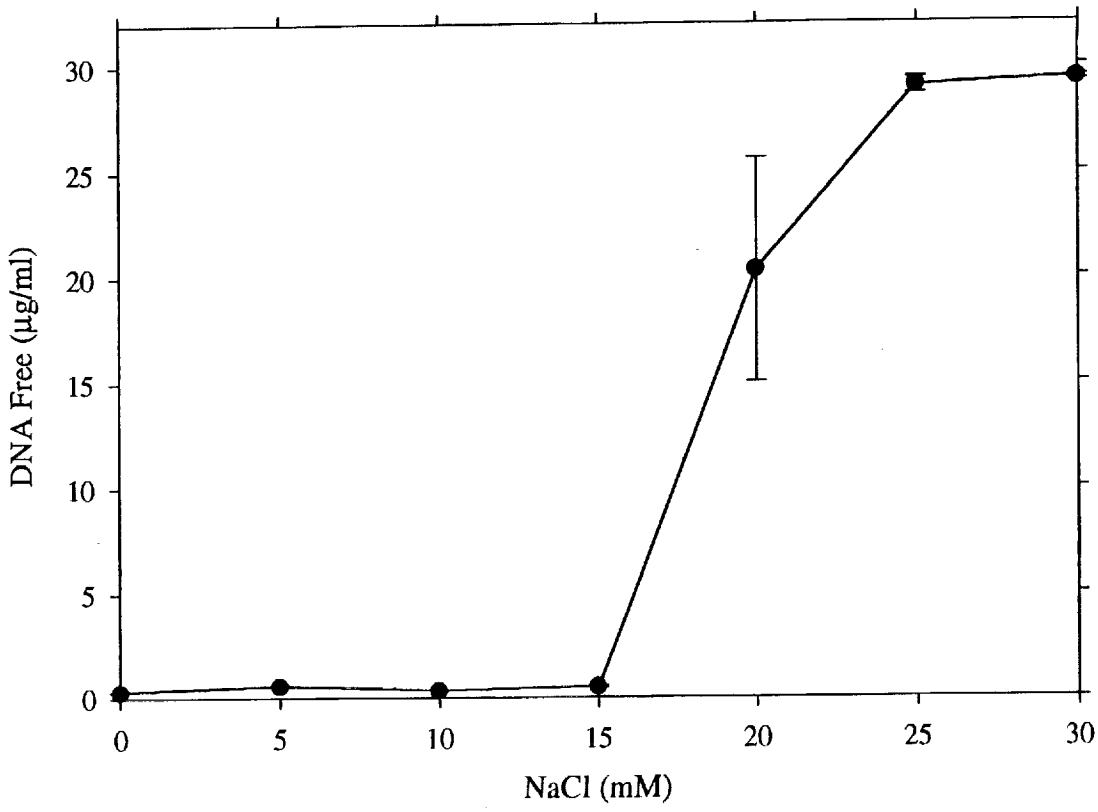


Figure 19

NaCl dependence of precipitation of pBGS19Luxwt (30 µg/ml) in 10 mM Tris, pH 8.0 buffer containing 1 mM spermidine. Error bars are +/- one standard deviation.

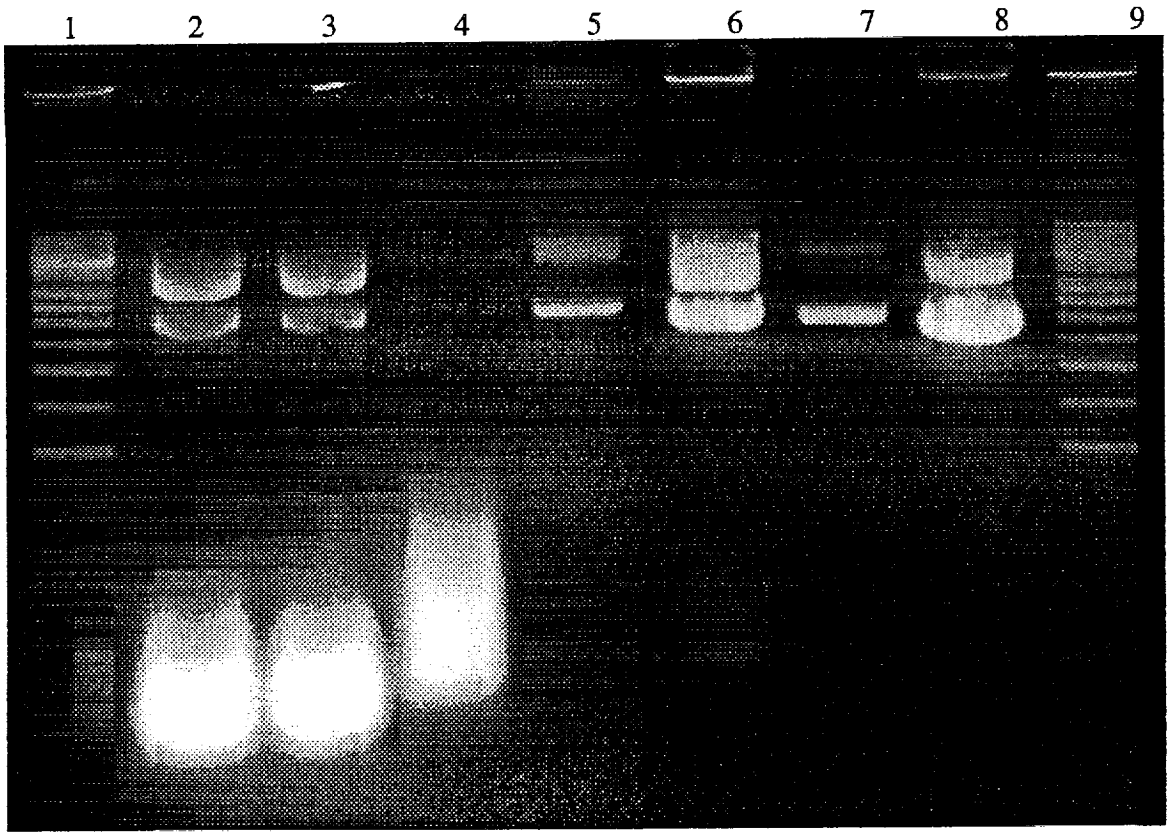


FIGURE 20:
1% agarose gel tracing the large-scale purification of pBGS19luxwt plasmid DNA. Lane 1 is a supercoiled plasmid ladder from Gibco; Lane 2 is the preparation after Celite filtration, isopropanol precipitation, and resuspension; Lane 3 is the supernatant after LiCl precipitation; Lane 4 is the supernatant of the compaction precipitation by 2.9 mM Spermidine HCl; Lane 5 is the resuspended pellet of the compaction precipitation after stripping of spermidine by 300 mM NaCl, 10 mM $MgCl_2$, and 25 mM EDTA in 50% isopropanol; Lane 6 is a 10X loading of the material in Lane 5 (we believe that the traces of genomic DNA in these lanes could be removed by further optimization of the initial lysis and precipitation steps); Lane 7 is after a Q Sepharose anion exchange column (Figure 4, bottom, Peak 5); Lane 8 is a 10X loading of Lane 7 and Lane 9 is a supercoiled plasmid ladder from Gibco.

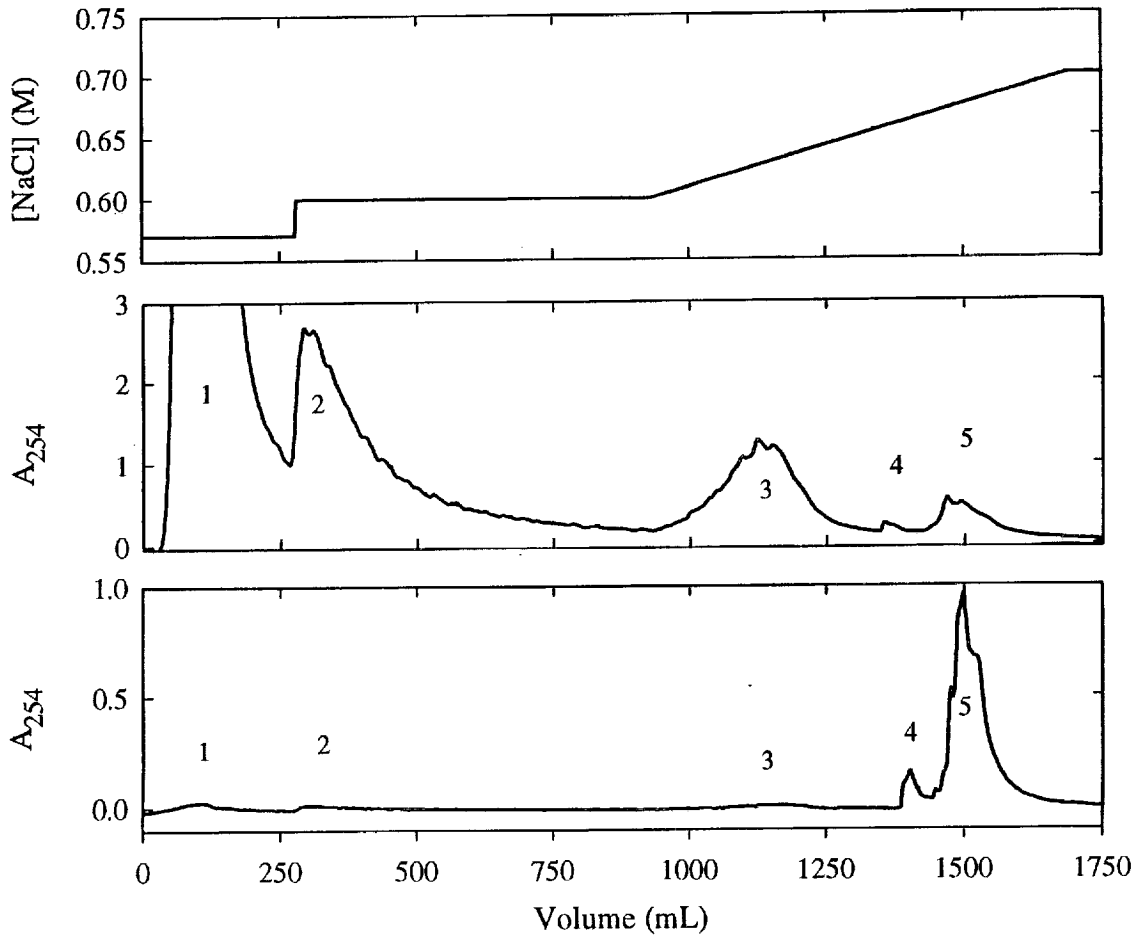


FIGURE 21: FPLC anion-exchange separation of pBGS19Luxwt of an alkaline lysate after isopropanol and LiCl precipitation. Top: NaCl gradient; Middle: with no previous compaction precipitation step; Bottom: identical separation after a previous compaction precipitation step. We used a Spectrum column (2.5 cm x 60 cm) packed with 150 mL Q Sepharose high performance and equilibrated in 10 column volumes of TE with 570 mMNaCl. Loading and elution were performed at a linear velocity of 90 cm/hr.

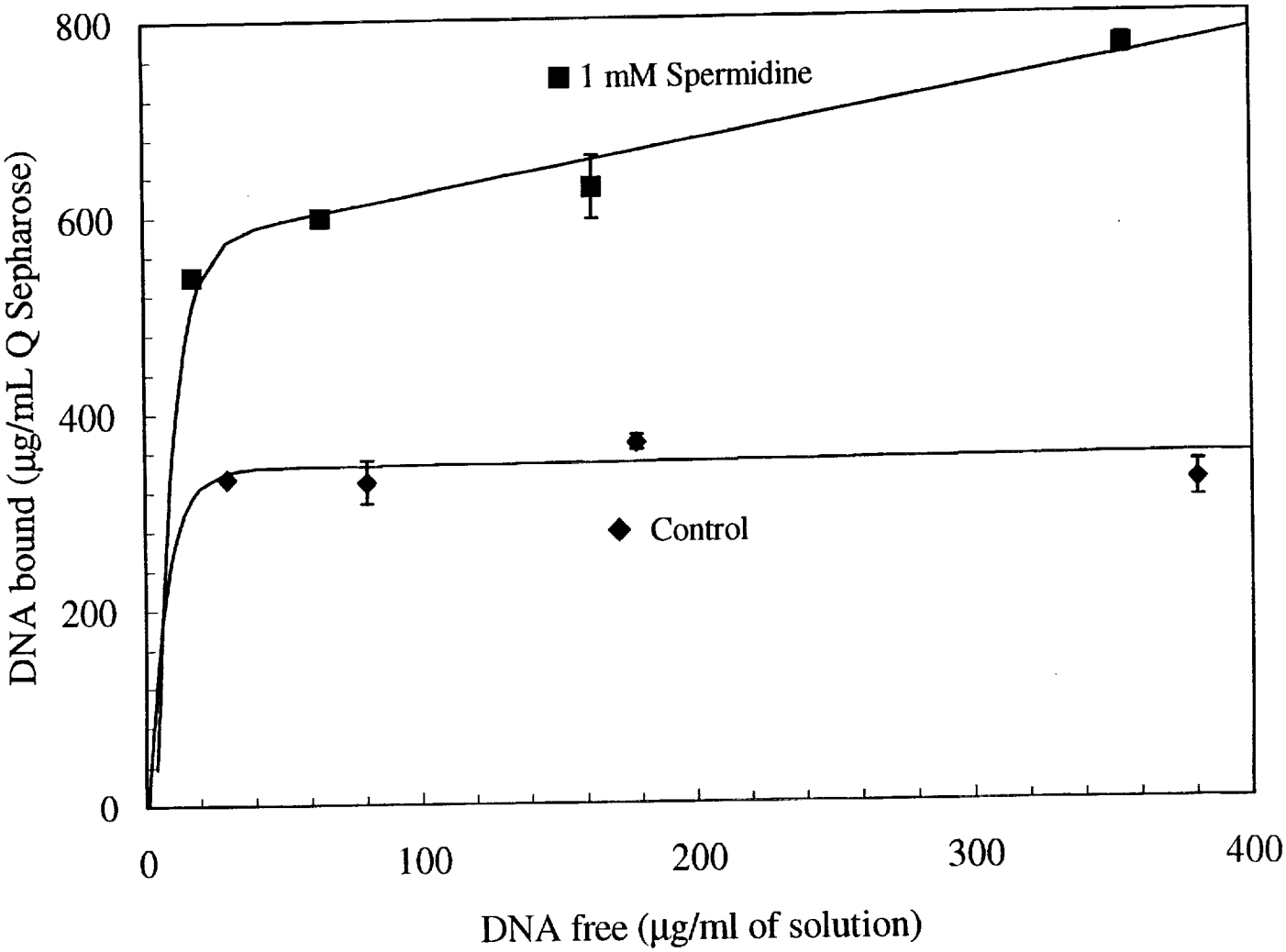


Figure 22: Equilibrium adsorption isotherm for DNA from salmon testes on Q Sepharose of in 10 mM Tris HCl, pH 8.0 with 75 mM NaCl, with or without 1 mM spermidine

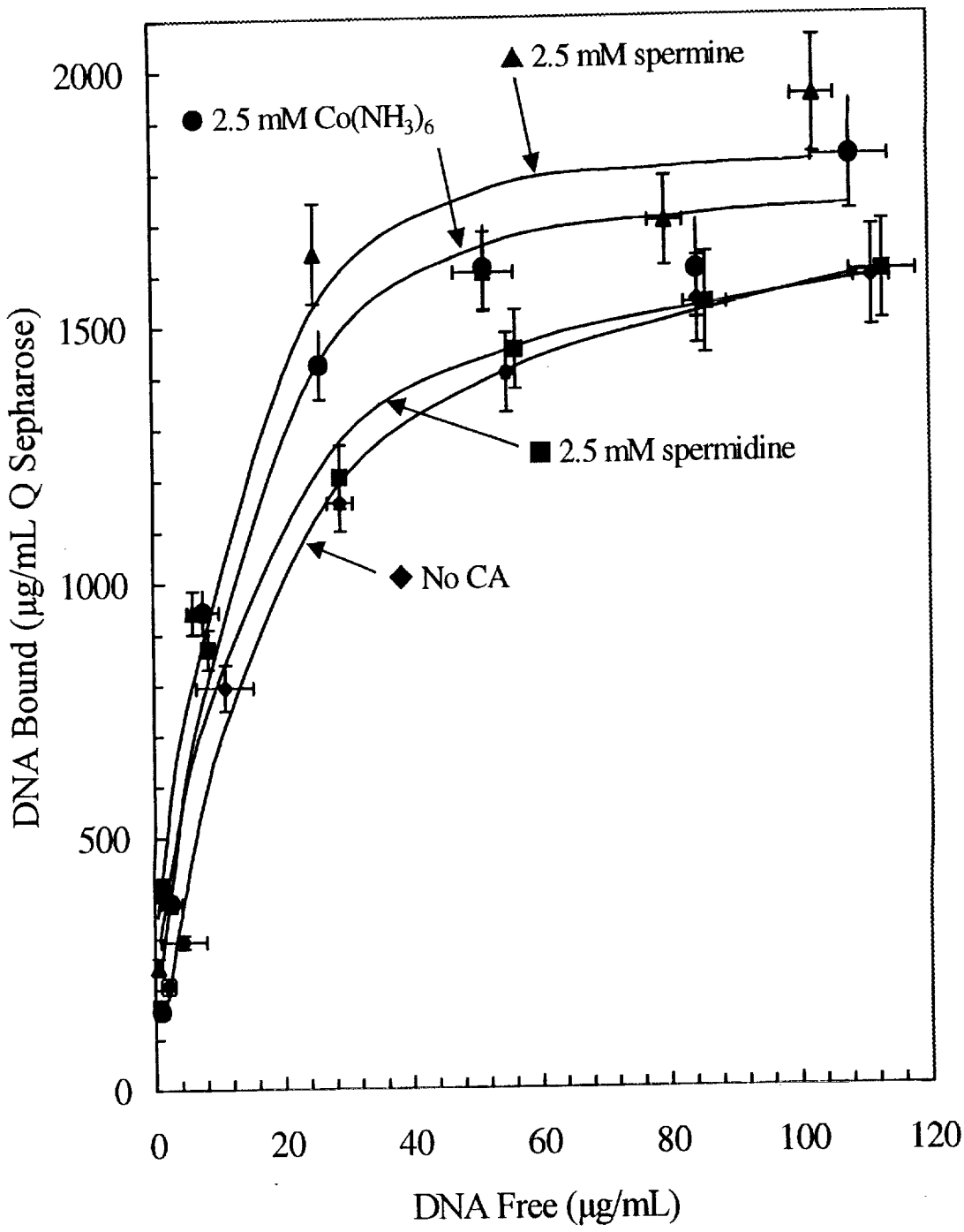


Figure 23: Salmon Sperm DNA adsorption on Q Sepharose in the presence of various compaction agents at 2.5 mM in 10 mM Tris HCl, pH 8.0 with 600 mM NaCl.

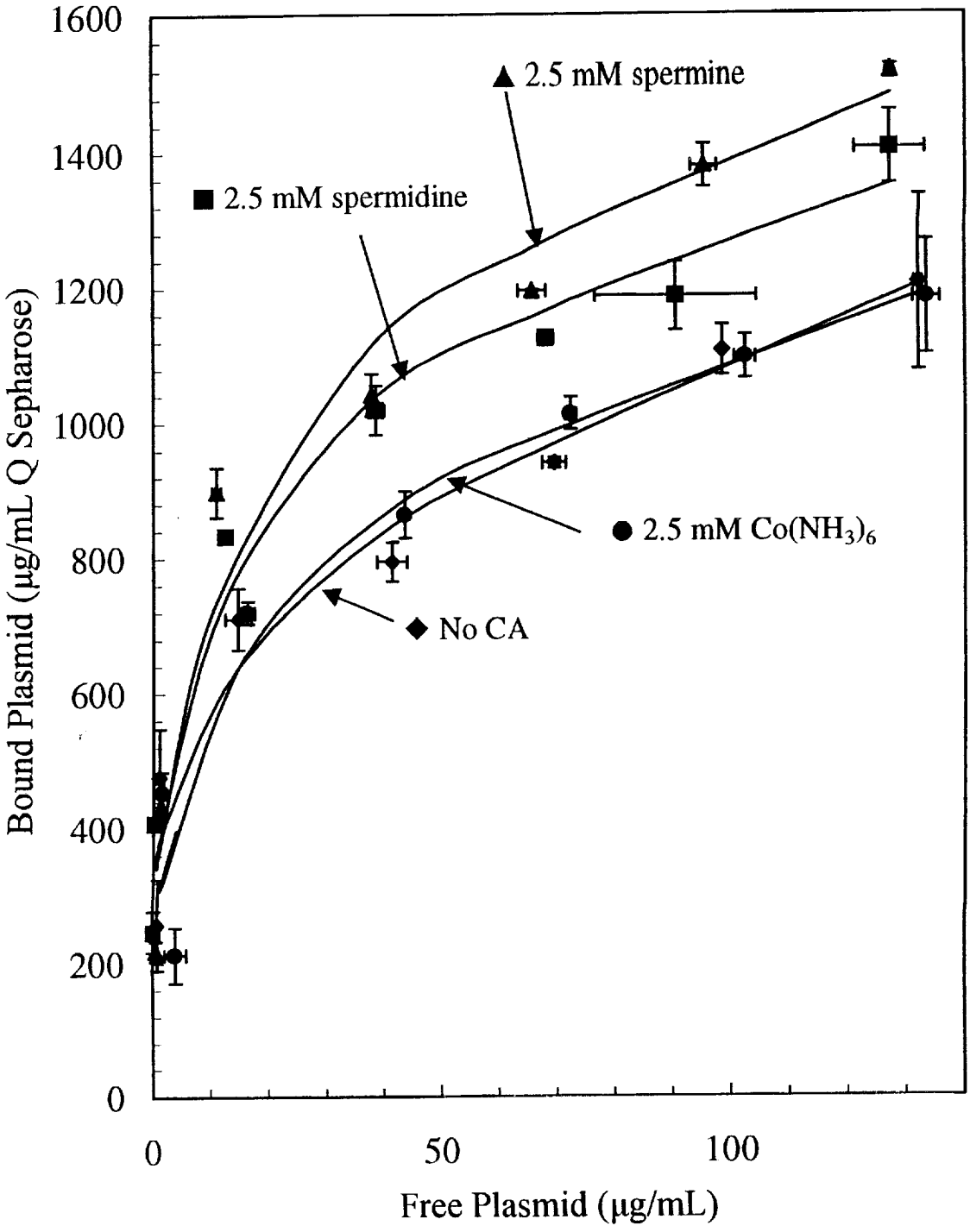


Figure 24: pBGS19luxwt binding to Q Sepharose in the presence of various compaction agents at a 2.5 mM concentration with 600 mM NaCl in 10 mM Tris at pH 8.0.

Figure 25: Qualitative summary of adsorption results

DNA Type	NaCl Conc. (mM)	Compaction agent (2.5 mM)	Effect
DNA from salmon testes	600	Spermidine	No Effect
	600	Spermine	Enhanced Binding
	600	$Co(NH_3)_6$	
	300	Spermidine	No Effect
	300	Spermine	
	300	$Co(NH_3)_6$	
	100	Spermidine	No Effect
	100	Spermine	Precipitation
	100	$Co(NH_3)_6$	
pBGS19luxwt	600	Spermidine	Enhanced Binding
	600	Spermine	
	600	$Co(NH_3)_6$	No Effect
	300	Spermidine	Enhanced Binding
	300	Spermine	
	300	$Co(NH_3)_6$	No Effect
	100	Spermidine	Enhanced Binding
	100	Spermine	Precipitation
	100	$Co(NH_3)_6$	

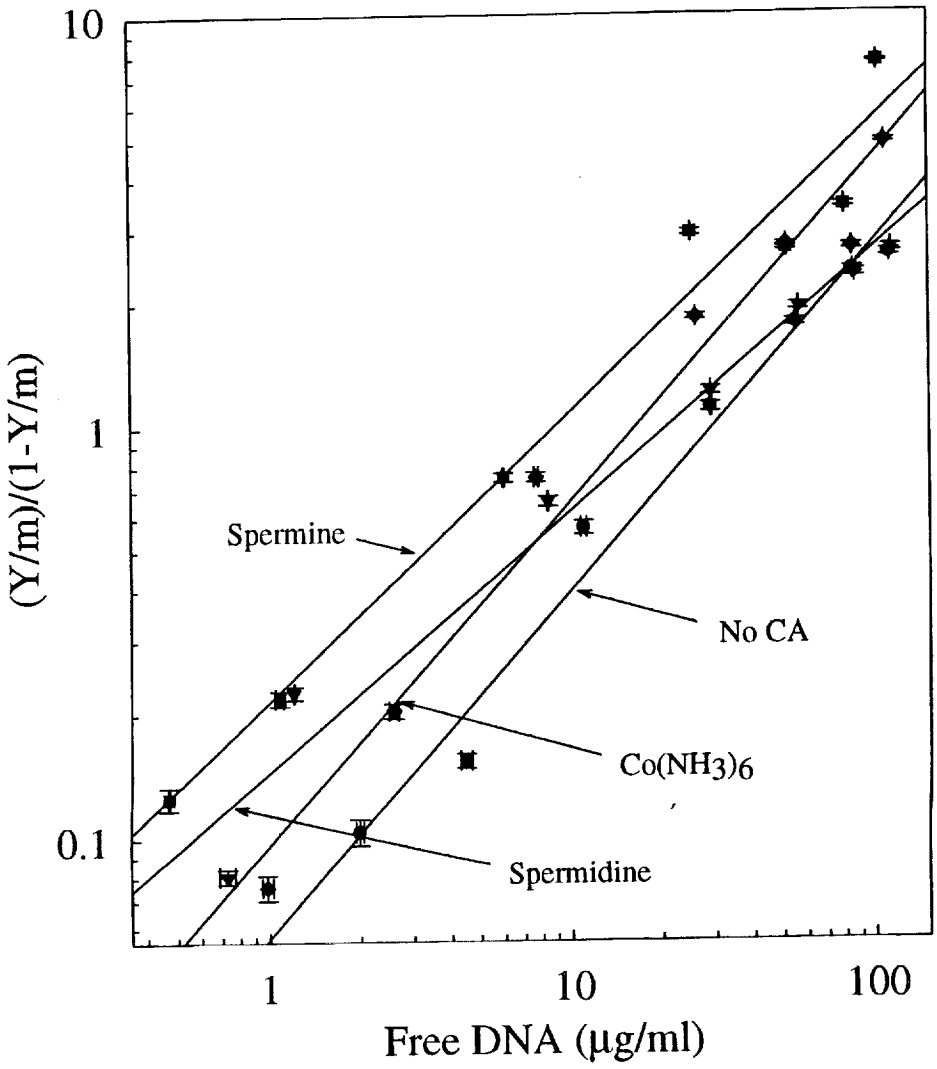


Figure 26: Hill Plot for adsorption of DNA from salmon testes on Q Sepharose in 600 mM NaCl. Y stands for µg of DNA bound per ml of Q Sepharose, and m is the ultimate DNA binding capacity of the matrix (µg/ml).

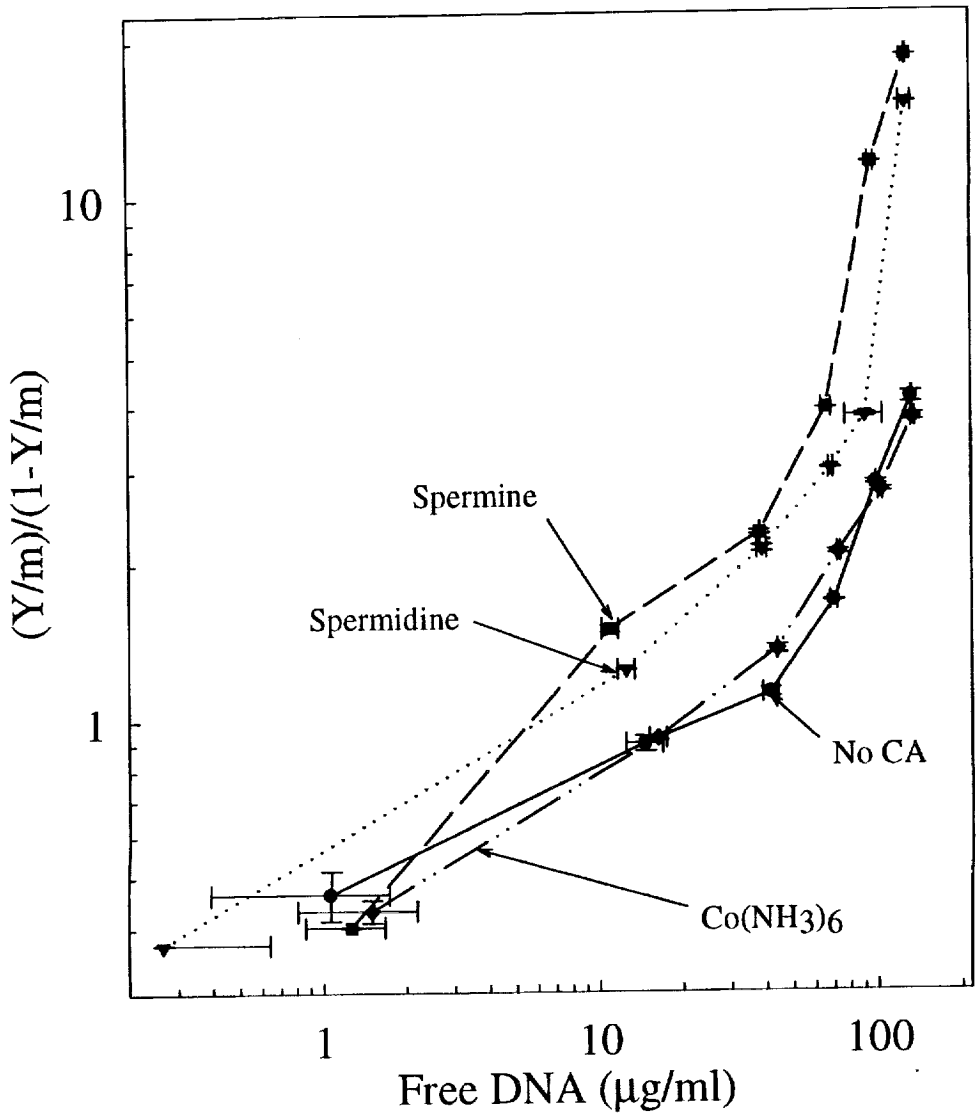


Figure 27: Hill Plot for adsorption of pBGS19luxwt on Q Sepharose in 600 mM NaCl. Y stands for µg of DNA bound per ml of Q Sepharose, and m is the ultimate DNA binding capacity of the matrix (µg/ml).

FIGURE 28:

A) The Hill constants and percent binding increase with various compaction agents for the DNA from salmon testes system.

(A)	NaCl (mM)	Comp. Agent	$K_D \pm$ std. dev.	% K_D increase ± std. dev.	$n_H \pm$ std. dev.
DNA from salmon testes	600	none	1.36±0.02	------	0.92±0.01
	600	spermidine	1.16±0.02	15±3	0.69±0.01
	600	spermine	0.78±0.07	42±7	0.83±0.07
	600	$Co(NH_3)_6$	1.07±0.02	21±3	0.95±0.02
	300	none	2.00±0.09	-----	0.90±0.04
	300	spermidine	2.04±0.03	-2±9	0.73±0.01
	300	spermine	2.02±0.01	-1±9	0.78±0.00
	300	$Co(NH_3)_6$	2.10±0.05	-5±10	0.55±0.01
	100	none	2.70±0.15	-----	0.47±0.03
	100	spermidine	2.52±1.26	7±127	0.44±0.22
	100	spermine	Precipitated		
	100	$Co(NH_3)_6$	Precipitated		

FIGURE 29:

B) The Hill constants and percent binding increase with various compaction agents for the pBGS19luxwt system.

(B)	NaCl (mM)	Comp. Agent	K_{50} ± std. dev.	% K_{50} increase ± std. dev.	n_{50} ± std. dev.
pBGS19luxwt	600	none	1.3±0.1	-----	1.1±0.1
	600	spermidine	0.8±0.1	36±5	1.1±0.1
	600	spermine	0.8±0.2	36±9	1.3±0.1
	600	$Co(NH_3)_6$	1.3±0.1	-----	1.1±0.1
	300	none	1.8±0.1	-----	1.0±0.1
	300	spermidine	1.7±0.1	3±5	1.0±0.1
	300	spermine	1.4±0.2	20±9	1.0±0.1
	300	$Co(NH_3)_6$	1.6±0.1	9±5	1.0±0.1
	100	none	2.1±0.1	-----	1.0±0.1
	100	spermidine	1.8±0.1	15±5	1.0±0.1
	100	spermine	Precipitated		
	100	$Co(NH_3)_6$	Precipitated		

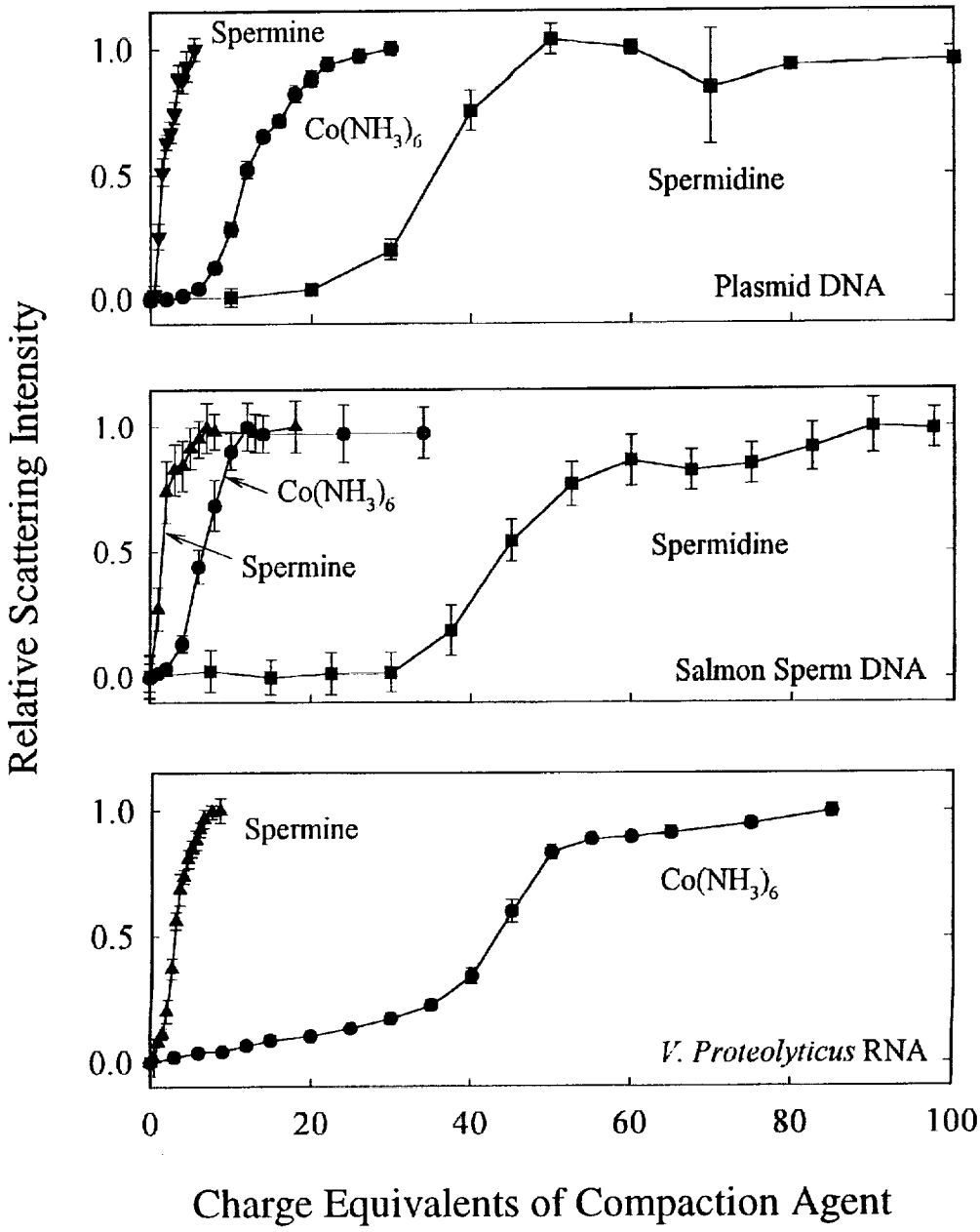


Figure 30: Light scattering- monitored compaction experiments. 10 μg/ml nucleic acid at 20°C in 10 mM bis tris propane buffer at pH 7.0. Top: plasmid DNA (pCMV sport β gal) in various compaction agents, Middle: salmon sperm DNA in various compaction agents, Bottom: *V. proteolyticus* total RNA in various compaction agents (spermidine was omitted from the *V. proteolyticus* total RNA plot as condensation did not occur up to 700 charge equivalents.).

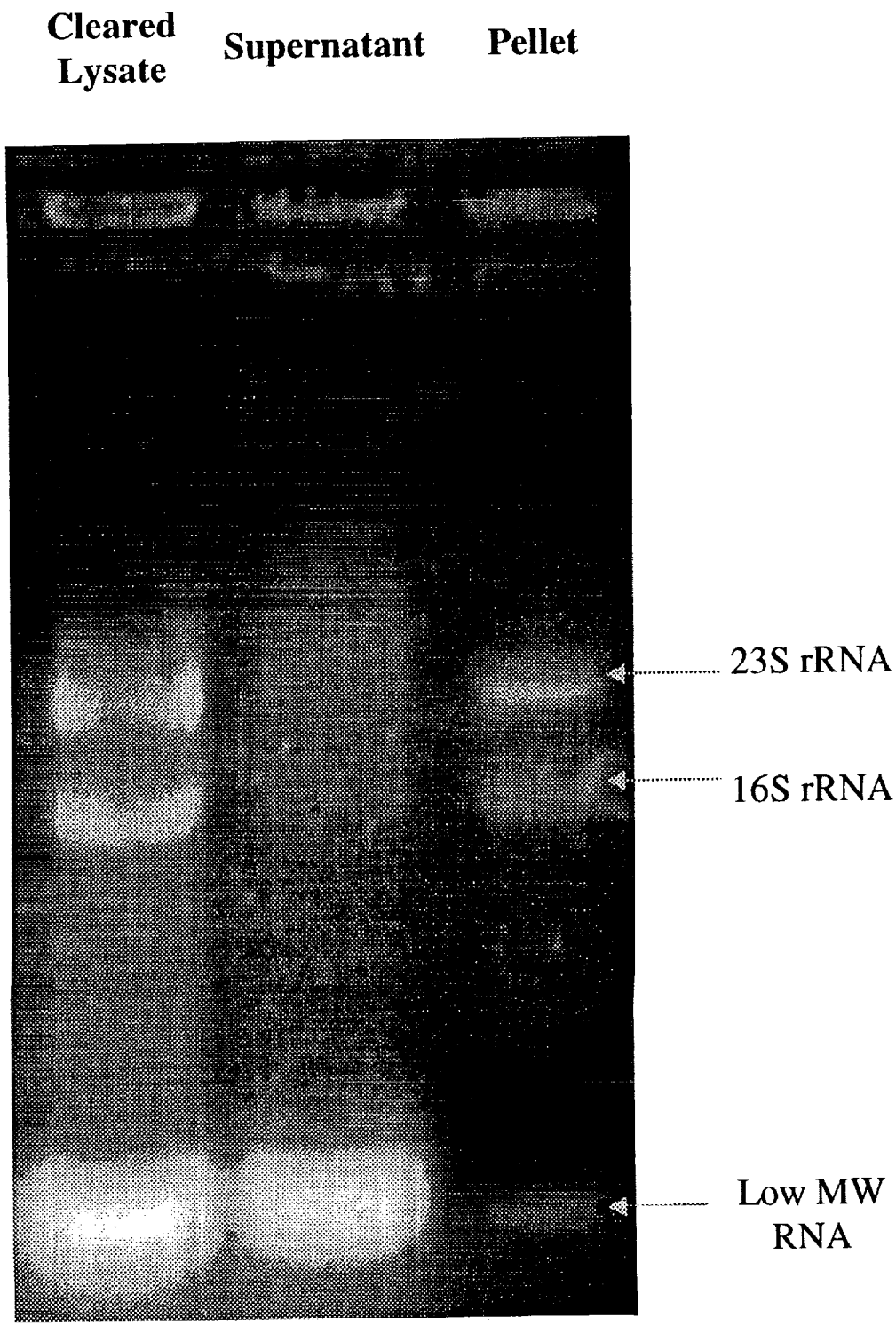


FIGURE 31: *V. proteolyticus* RNA separation by hexammine cobalt precipitation. Lane 1 is RNA Century ladders from Ambion, Lane 2 is the BPER/spermidine initial lysate, Lane 3 is the supernatant of the 2 mM hexamine cobalt RNA precipitation, and Lane 4 is the resupended and compaction agent stripped pellet of the hexamine cobalt precipitation.

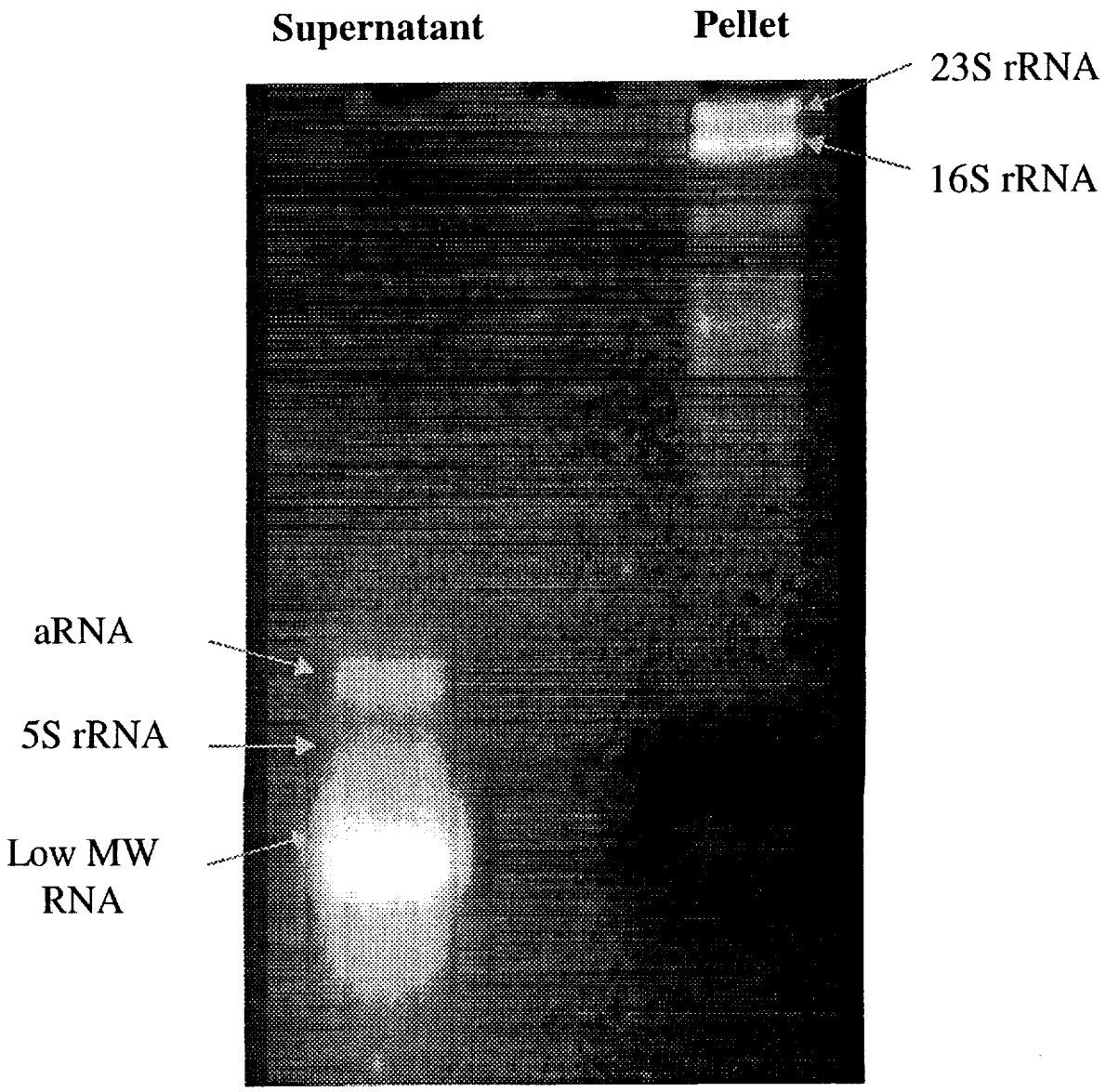


FIGURE 32: Separation of pCP3X3 artificial RNA by hexamine cobalt fractionation. Lane 1 is the supernatant of the 2 mM hexamine cobalt RNA precipitation and Lane 2 is the resuspended and compaction agent stripped pellet of the 2 mM hexamine cobalt precipitation.

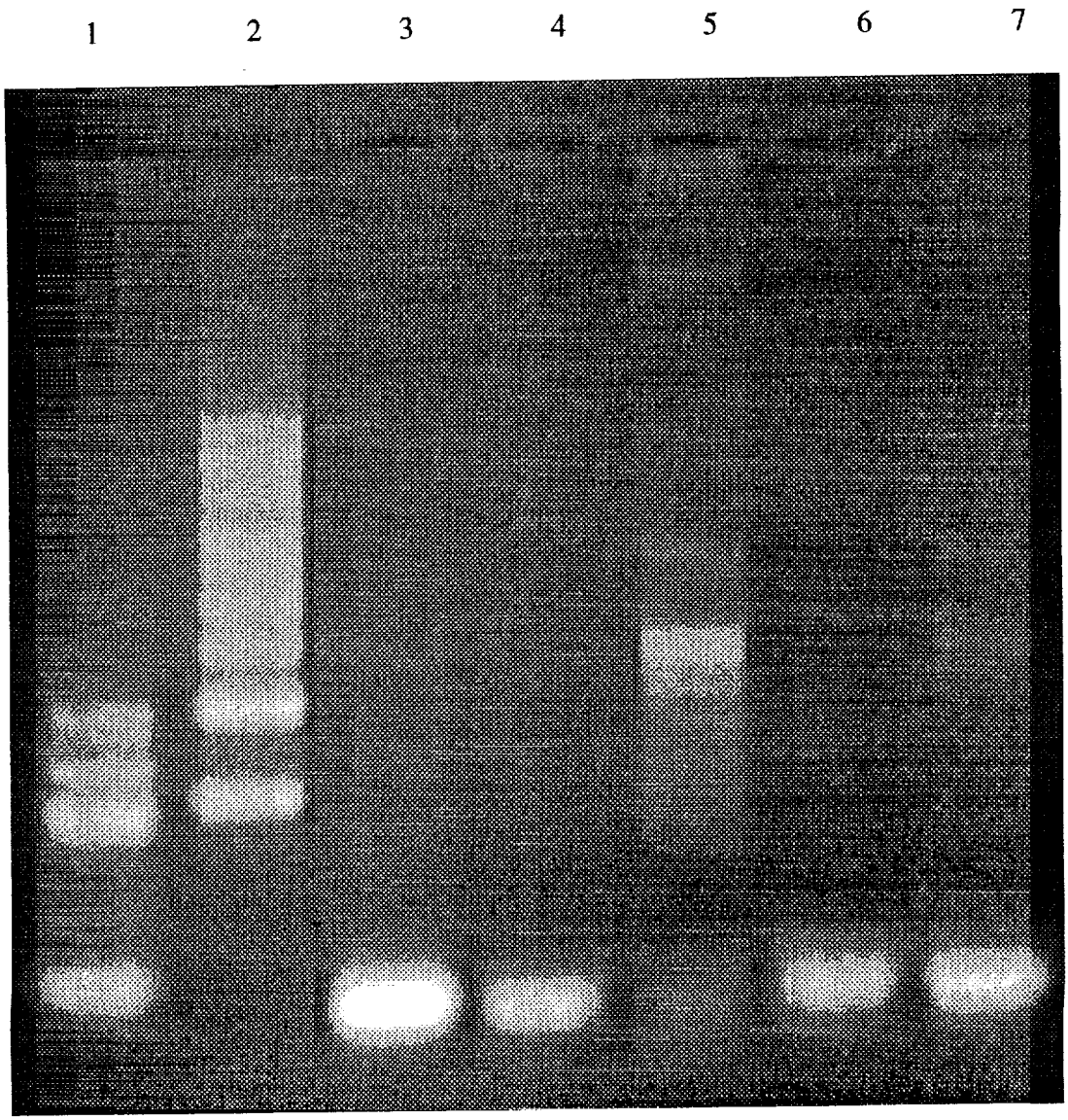


FIGURE 33: 2% agarose gel showing each step in the three-stage β ribozyme compaction precipitation protocol. Lane 1 is RNA Century ladders from Ambion (100-500 bases), Lane 2 is the RNA Millennium ladders from Ambion (0.5-9 kilobases), Lane 3 is the initial lysate, Lane 4 is the pellet of the first compaction precipitation (with 2 mM hexammine cobalt), Lane 5 is the supernatant of the first precipitation, Lane 6 is the supernatant of the second precipitation with 7.1 mM hexammine cobalt, and Lane 7 is the pellet of the second precipitation.

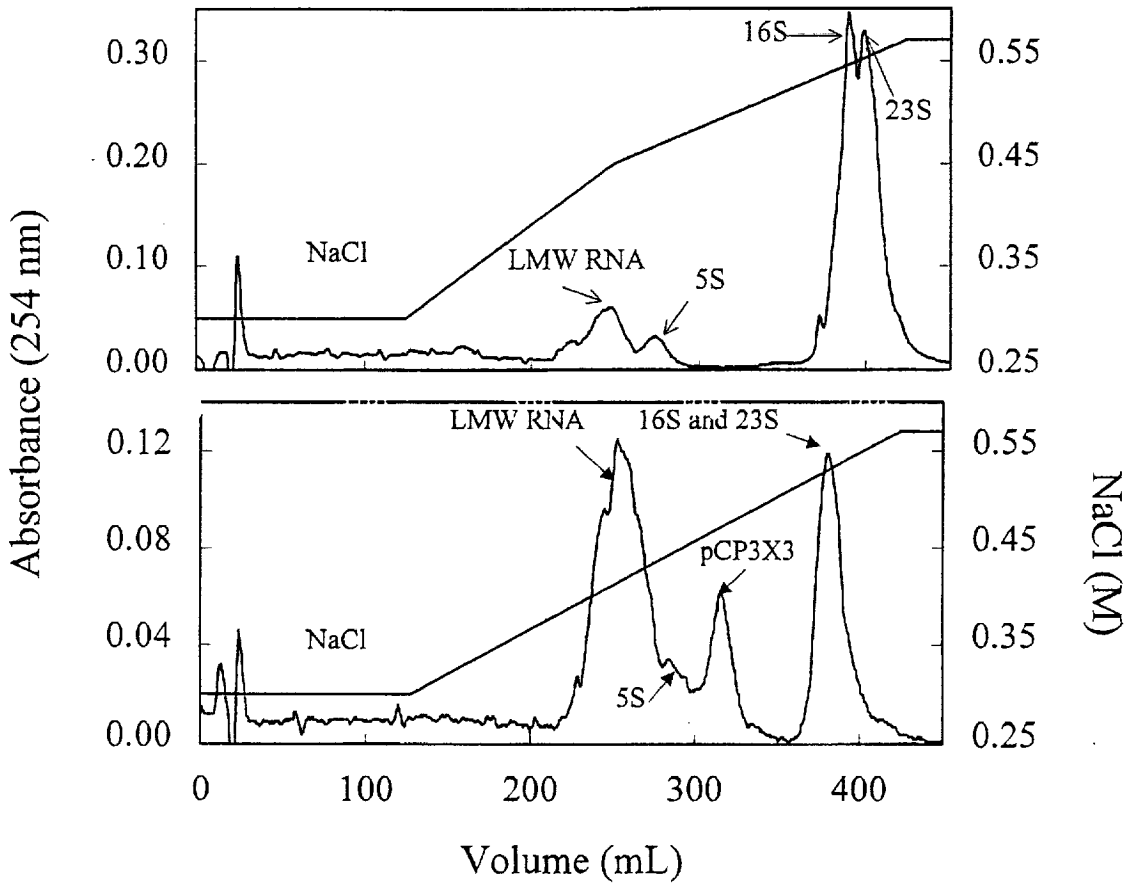


FIGURE 34

Multiple FPLC chromatograms from nondenaturing anion-exchange chromatography of RNA. Top: Chromatogram of *V. proteolyticus* RNA on a 10 ml high performance Q Sepharose anion-exchange column (Pharmacia). The gradient was run over 12 column volumes from 0.30 M NaCl to 0.57 M NaCl in a column buffer of 20 mM bis-tris propane and 20 mM EDTA at pH 6.9. Bottom: same as A except the aRNA pCP3 X3 in *E. coli* JM109 was purified.

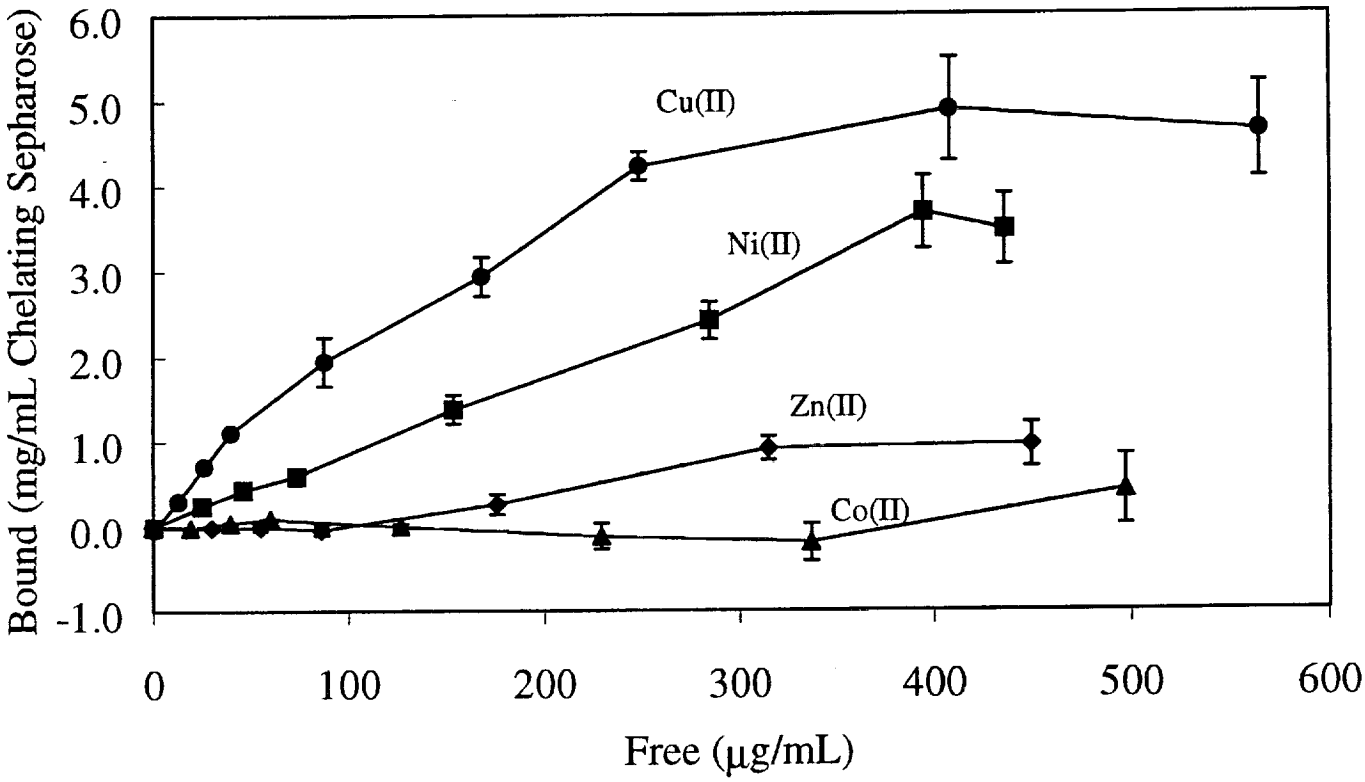


Figure 35: Different Isothermal binding curves for different nucleic acids interacting with X (II) charged IDA Chelating Sepharose matrix in 10 mM HEPES with 250 mM NaCl at pH 7.0. Metal ions commonly used in IMAC applications, namely Cu (II), Ni (II), Zn (II), and Co (II), to show the different affinities of each metal chelate toward bakers yeast RNA.

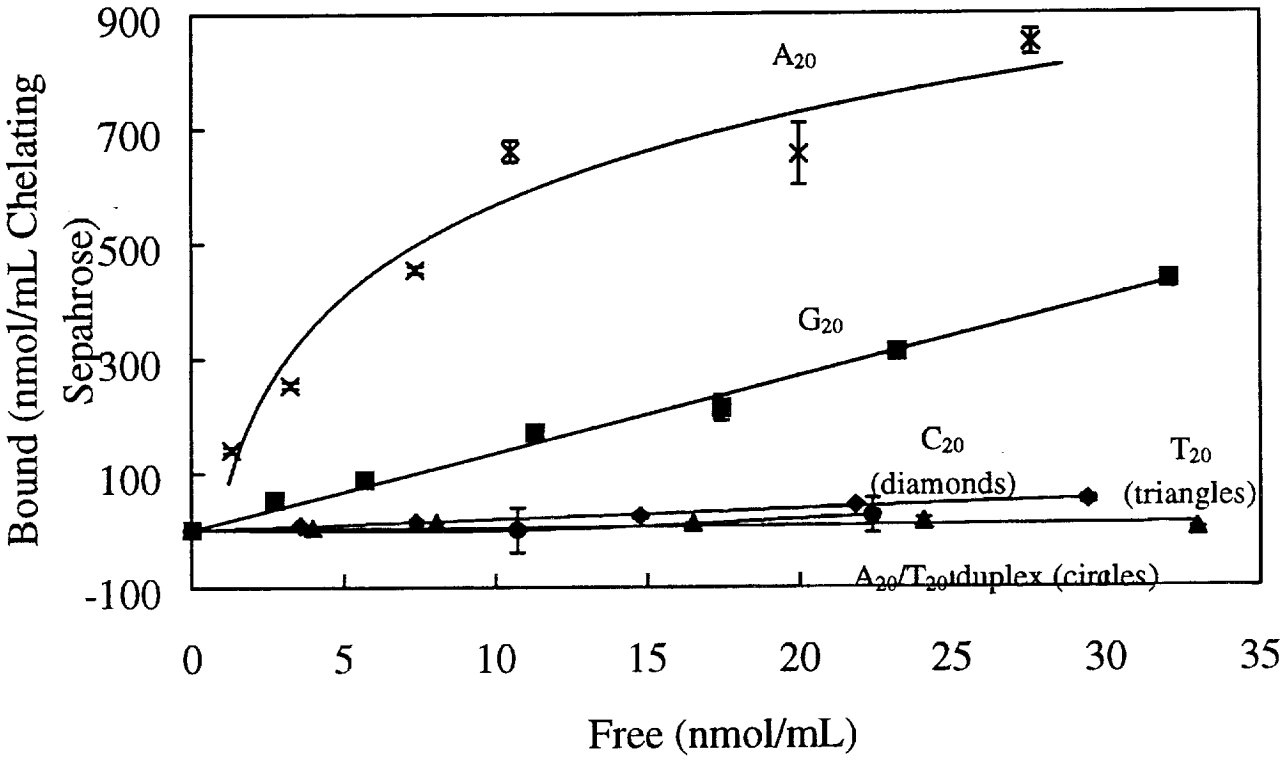


FIGURE 36: Isotherms of 20-mer homopolymer oligonucleotides showing the different affinities of each base (A, G, C, and T) toward a Ni (II) charged IDA Chelating Sepharose matrix in 10 mM HEPES with 250 mM NaCl at pH 7.0.

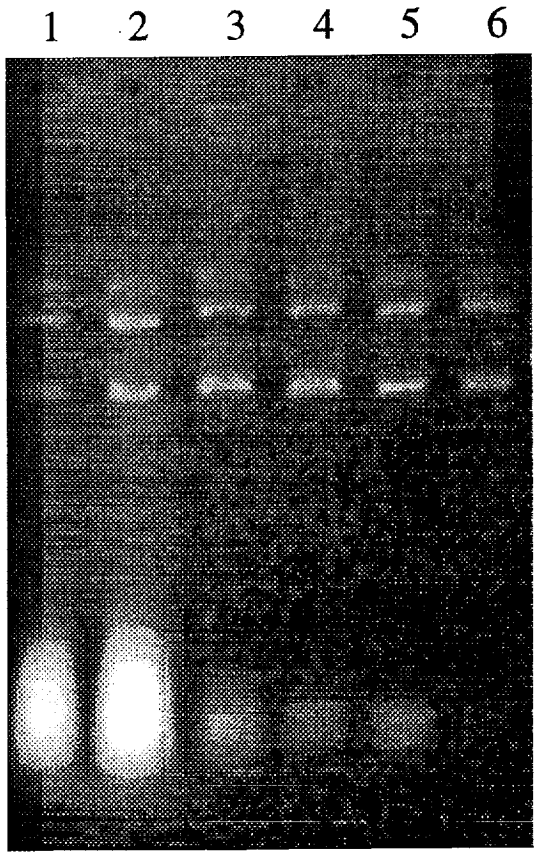


FIGURE 37: Repeated Cu IDA stripping of RNA from plasmid. EtBr stained 1% agarose gel of Cu (II) charged Chelating Sepharose matrix batch adsorption experiment of alkaline lysed *E. coli* with plasmid pBGS19luxwt. Lane 1 is the original lysate; Lane 2 is lysate contacted with non-charged IDA matrix; Lane 3 is the unbound material after a single batch adsorption; Lane 4 is Lane 3 after exposure to fresh matrix, Lane 5 similarly is Lane 4 after exposure to fresh matrix and Lane 6 is Lane 5 after exposure to fresh matrix.

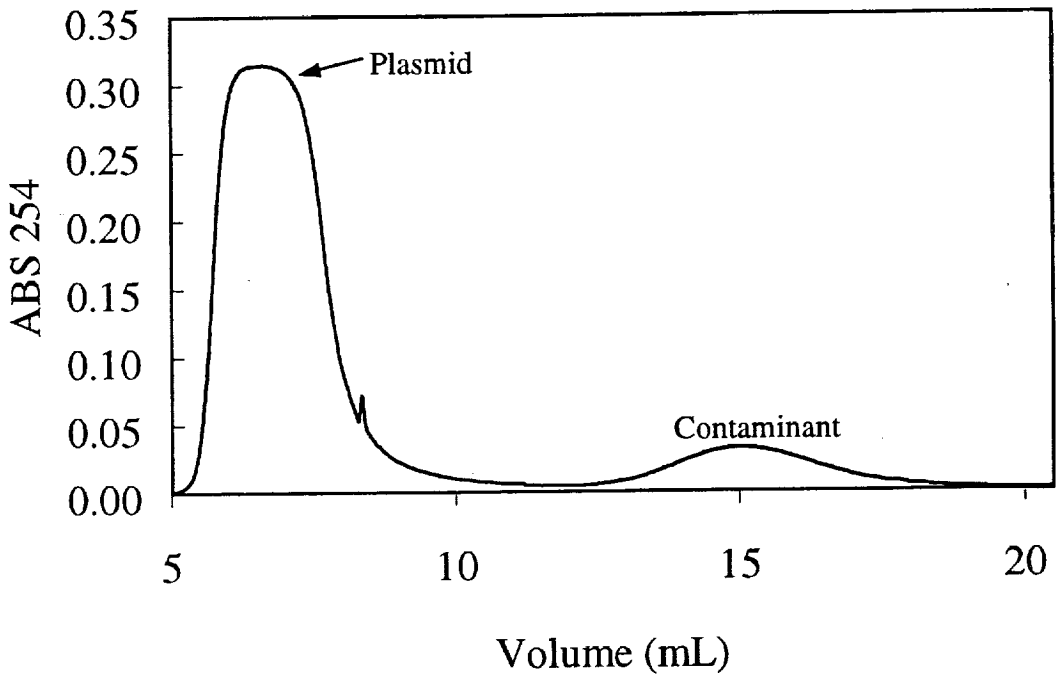


FIGURE 38: Plasmid separation on Cu (II) charged IMAC. The FPLC chromatogram show the plasmid pCMV sport β gal ran over a 20 mL Cu (II) charged IMAC column (1 X 15 cm Amicon FPLC column packed with Chelating Sepharose Fast Flow) in column running buffer (no gradient). The plasmid passed through the column with no hold-up while RNA and other damaged nucleic acids binding to the IMAC media or some unwanted contaminants where held up on the column and were retained longer (isocratic separation). Nucleic acids were detected by gel electrophoresis and RNA was not visible on the 0.8 agarose gels run.

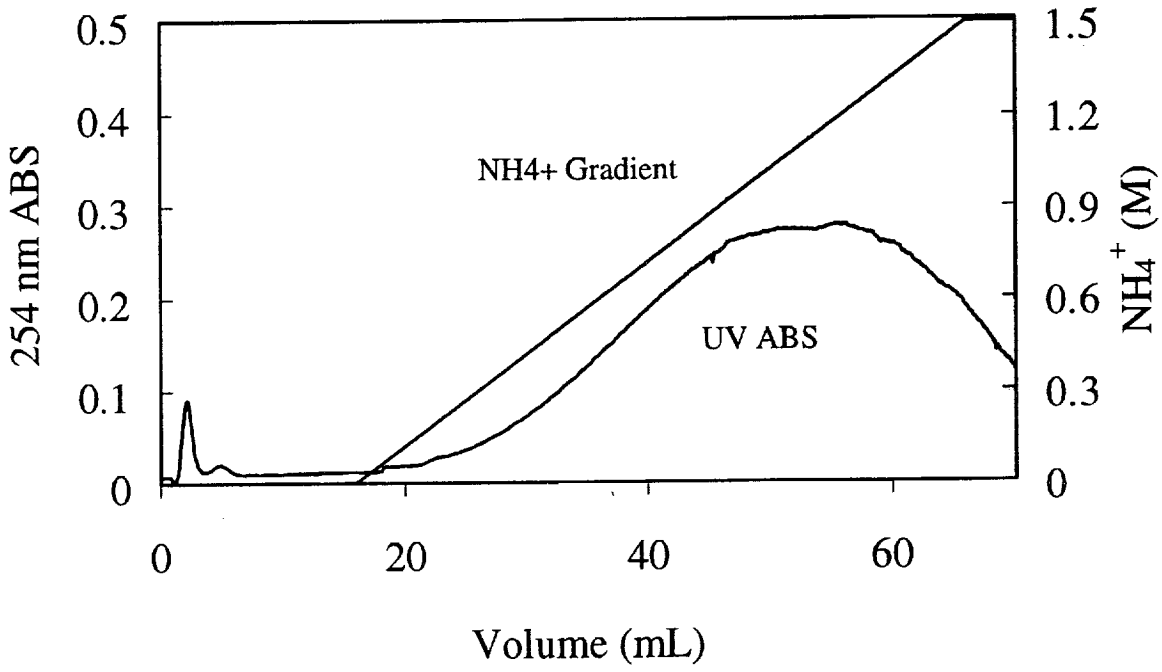


FIGURE 39: FPLC Chelating Sepharose separation of β ribozyme after compaction precipitation. The FPLC chromatogram traces the binding of β ribozyme to a 2 mL HyTrap Chelating Sepharose Column (2 chelating Sepharose 1 mL columns in series) and the subsequent elution. The ribozyme was loaded in column running buffer and a gradient was run from 0 to 1.5 M NH_4Cl.

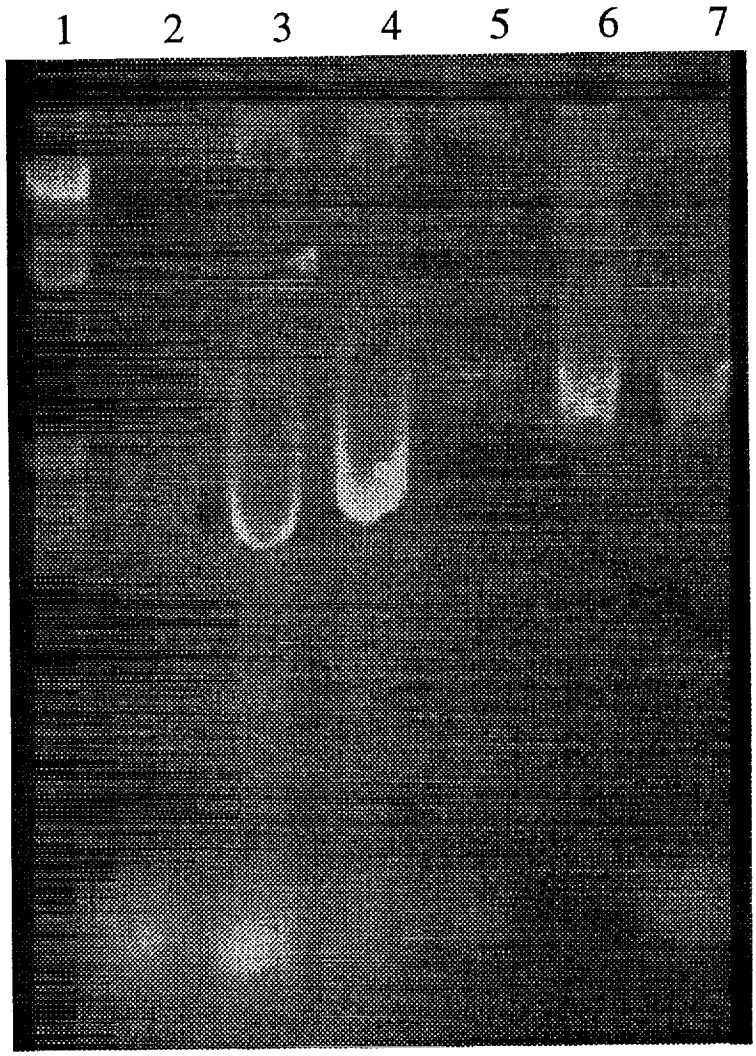
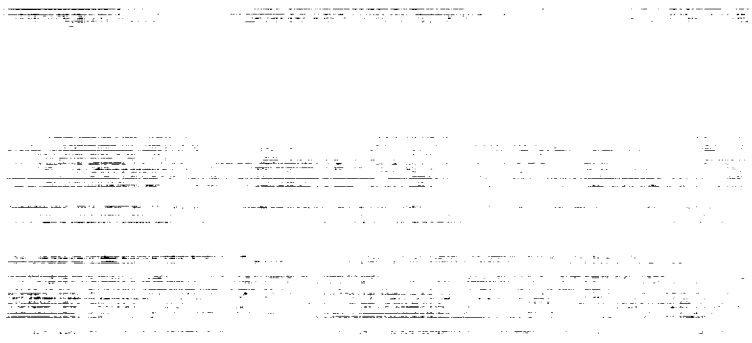


FIGURE 40: 2% agarose gel stained with SYBR Gold nucleic acid stain (Molecular Probes) of PCR product cleanup. Lane 1 is the 1 kb ladder; Lane 2 is PCR primers (forward and reverse) for the plasmid pCMV sport b gal (Gibco); Lane 3 is an PCR reaction amplifying an ~ 800 bp fragment of pCMV sport b gal; Lane 4 is the unpurified PCR product ran through a Ni (II) charged spin column; Lane 5 is the elution of the Ni (II) charged spin column from Lane 4 (eluted with 500 mM imidazol in column running buffer); Lane 6 is the unpurified PCR product ran through a Cu (II) charged spin column; and Lane 7 is the elution of the Cu (II) charged spin column from Lane 6 (eluted with 500 mM imidazol in column running buffer).



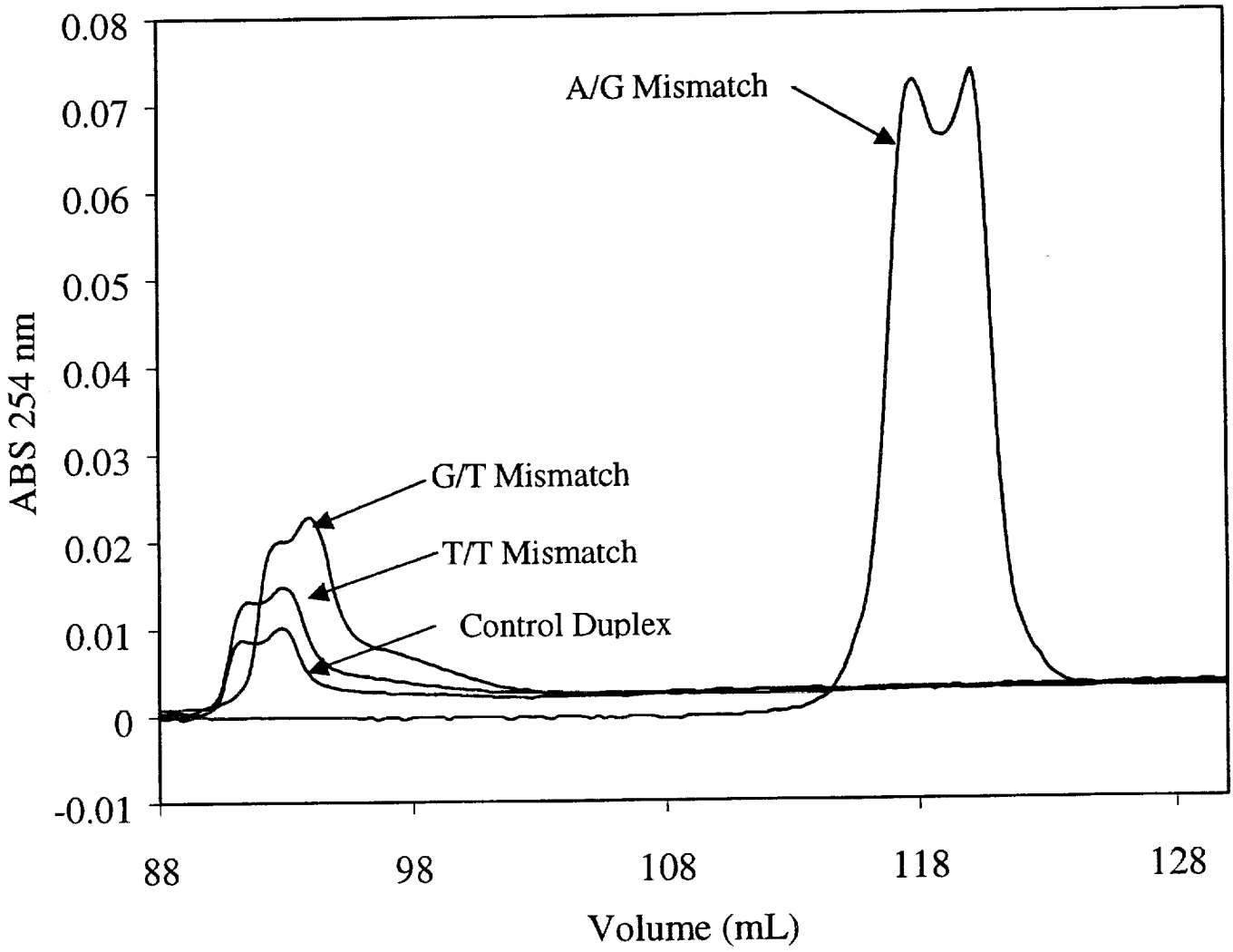


FIGURE 41: FPLC gradient separation of 20 mer duplexed oligos with and without SNP's. The column used was a 1 cm X 15 cm Amicon packed with Chelating Sepharose Fast Flow media charged with Cu(II). The running buffer was 20 mM HEPES with 250 mM NaCl at pH 7.0. This initial gradient was over 10 column volumes from 0 to 50 mM imidazol. The plot above shows a 2 column volume portion of 4 separate FPLC runs.

APPENDIX B-
PUBLICATIONS, PATENTS AND PRESENTATIONS

1. Publications

Singh, N. and Willson, R. C., "Boronate Affinity Adsorption of RNA: Possible Role of Conformational Changes", *J. Chromatogr. A,* **840:** 205-213 (1998).

Murphy, J. C., Wibbenmeyer, J. A., Fox, G. E., and Willson, R. C., "Nucleic Acid Purification Using Compaction Agents", *Nature Biotechnology,* **17:** 822-823 (1999).

Larios-Sanz, M., Kourentzi, K. D., Murphy, J. C., Maillard, K. I., Pearson, D. L., Willson, R. C., Fox, G. E. , "Monitoring Microbial Populations in Space Environments", in: " Dianostico Molecular" JGH Editores SA de CV, Mexico City, Mexico, (2000), in press.

Kourentzi, K. D., Fox, G. E., and Willson, R. C. "Rapid Detection of Microorganisms Using 5S rRNA-Specific Molecular Beacons", submitted to *Environ. Sci. Technol.*, 9/2000.

Murphy, J. C., Fox, G. E., and Willson, R. C. "Structured RNA Isolation and Fractionation with Compaction Agents", submitted to *Nucleic Acids Res.* 9/2000.

Murphy, J. C., Fox, G. E., and Willson, R. C. "Enhancement of Anion Exchange Separation of DNA Using Compaction Agents", in preparation

Murphy, J. C., Jewell, D. L., White, K. I., Fox, G. E., and Willson, R. C. "Separation of Nucleic Acids Using Immobilized Metal Affinity Chromatography", in preparation.

2. Patent Filings

Murphy JC and Willson, RC, Methods and compositions for biotechnical separations using selective precipitation by compaction agents, patent pending.

Murphy JC and Willson, RC, Methods and compositions for nucleic acid separations using immobilized metal affinity agents. patent application in preparation.

3.Presentations

Wibbenmyer, J. A., Larios-Sanz, M., Willson, R. C. and **Fox, G. E.,** "DNA Probe Design for Preflight and Inflight Microbial Monitoring", National Space Biomedical Research Institute June Retreat, June 8-11, 1998, Del Lago Conference Center, Conroe, TX (poster presentation).

Murphy, J. C., Fox, G. E., and Willson, R. C., "Nucleic Acid Separations Using Compaction Agents", Poster Presented at 216th American Chemical Society Meeting, Abstract # BIOT-115, August 23-27, 1998, Boston, MA (poster presentation).

Walia, R. P., Murphy, J. C., Fox, G. E., and Willson, R. C., "Microbial Monitoring Using Hybridization Assays and Artificial RNA Labels", 216th American Chemical Society Meeting, Abstract # BIOT-223, August 23-27, 1998, Boston, MA (poster presentation).

Murphy, J. C. and Willson, R. W., "Nucleic Acids Separation Using Compaction Agents", Los Pines Molecular Biology Conference, Ocober 11, 1998, Smithville, Texas (poster presentation).

Wibbenmeyer, J. A., Larios-Sanz, M., Maillard, K., Pierson, D., Willson, R. C., and Fox, G. E. "Monitoring Bacteria in Space Environments", 14th Ann. Meeting Soc. Gravitational & Space Biol., October 28-31, 1998, Houston, TX (poster presentation), Abstract: *Gravitational & Space Biol. Bull.* **12:** 50 (1998).

Fox, G. E., Wibbenmeyer, J., Larios-Sanz, M., Kourentzi, K., Murphy, J. C., Willson, R. C., "Microbial Monitoring Technology for Long Duration Space Flights", 1st Biennial Space Biomedical Investigators' Workshop, Abstracts p198, January 11-13, 1999, League City, TX (oral presentation).

Kourentzi, K. D., Walia, R. P., Murphy, J. C., Wibbenmeyer, J. A., Larios-Sanz, M., Fox, G. E., and Willson, R. C., "Environmental monitoring of microorganisms using artificial rRNA labels and molecular beacons", 17th Annual Houston Conference on Biomedical Engineering, Feb. 11-12, 1999, Houston, TX (poster presentation).

Murphy, J. C., Fox, G. E., and Willson, R. C., "Nucleic Acids Separation Using Compaction Agents", 17th Annual Houston Conference on Biomedical Engineering, Feb. 11-12, 1999, Houston, TX (poster presentation).

Murphy, J. C., Wibbenmeyer, J. A., Fox, G. E., and Willson, R. C., "Nucleic Acids Separations using Compaction Agents", American Institute of Chemical Engineers 1999 Spring Meeting, March 14-18, 1999, Houston, TX (poster presentation).

Kourentzi, K.D., Walia, R. P., Murphy, J. C., Wibbenmeyer, J. A., Larios-Sanz, M., Fox, G. E., and Willson, R. C., "Environmental monitoring of microorganisms using artificial rRNA labels and molecular beacons", Abstract BIOT-088, 217th National Meeting, American Chemical Society, March 21-25, 1999, Anaheim, CA (poster presentation).

Murphy, J. C., Fox, G. E., and Willson, R. C., "RNA Separation using compaction precipitation", 217th National Meeting, American Chemical Society, Anaheim, CA., March 21-25, 1999 (oral presentation).

Kourentzi, K., Larios-Sanz, M., Setterquist, R. A., Fox, G. E., and Willson, R. C., "Environmental Monitoring Using rRNA Labels and Hybridization Assays", Gordon Conference

on Applied Environmental Biology, Connecticut College, Ct, July 3-8, 1999 (poster presentation).

Murphy, J. C., Fox, G. E., and Willson, R. C., "Nucleic Acid Separations Using Compaction Murphy, J. C., Fox, G. E., and **Willson, R. W.**, "Separation of Nucleic Acids Using Compaction Agents", 13th Intn. Symp. Affinity Technology & Recognition, July 1999, Compiegn, France, (invited oral presentation).

Murphy, J. C., Fox, G. E., and Willson, R. W. , "Nucleic Acids Separation Using Compaction Agents", Abstract BIOT-, 218th National Meeting American Chemical Society, August 26, 1999, Boston MA (poster presentation).

Murphy, J. C., White, K. I., Fox, G. E., and **Willson, R. W.**, " New Approaches to Nucleic Acid Separation.", Bioseparations Center, University BOKU, Vienna, Austria, February, 2000 (invited oral presentation).

Murphy, J. C. ,White, K. I., Fox, G. E., and Willson, R. C. "Nucleic Acid Separation Using Compaction Agents", Abstract BIOT-205, 219th National Meeting American Chemical Society, March 26-30, 2000, San Francisco, CA. (poster presentation).

Kourentzi, K. D., Fox, G. E., and Willson, R. C. "Monitoring Microorganisms Using Hybridization Assays Specific for rRNA", Abstract BIOT-279, 219th National Meeting American Chemical Society, March 26-30, 2000, San Francisco, CA. (poster presentation).

Murphy, J. C., White, K. I., Fox, G. E., and **Willson R. C.** ."New approaches to nucleic acid separation", Tenth International Symposium on Analysis and Separation of Proteins, Peptides and Polynucleotides, Ljubljana, Slovenia, November, 2000 (scheduled).

APPENDIX C

COPIES OF PUBLICATIONS (With Original Report Only)

APPENDIX C

COPIES OF PUBLICATIONS (With Original Report Only)

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE
2000 ANNUAL PROJECT REPORT

PROJECT TITLE: Neocytolysis: Mechanisms and Limitations

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Signature Clarence Alfrey Date 9/15/00

EXECUTIVE SUMMARY

Astronauts who go up into space have too much blood for their new environment. The hormone erythropoietin, which controls the production of red blood cells, quickly becomes suppressed. Our data from spaceflight demonstrates that there ensues destruction of young red blood cells less than 12 days old, allowing rapid adaptation. On re-entry, astronauts find themselves maladapted for function in a gravitational environment, being hypovolemic and anemic. To confirm our theory of neocytolysis, we studied high altitude residents who descend to sea level. Like astronauts entering microgravity they suddenly find themselves with excessive blood mass for their new environment. As predicted, erythropoietin levels fell and the number of red blood cells fell very quickly. Destruction of the red cells could be prevented by low doses of erythropoietin.

In our current studies, we are dissecting the mechanisms effecting neocytolysis, the selective destruction of young red blood cells. We have been able to demonstrate erythropoietin receptors exist on splenic endothelial cells. This is important because the spleen is the most likely site of neocytolysis. We have created an in vitro model of the process in which endothelial cells grow above macrophages. When the cells are grown in erythropoietin-containing medium and then have erythropoietin withdrawn, splenic endothelial cells become more permeable. They allow increased diffusion of large sugars and they allow increased phagocytosis of young RBCs by macrophages. Young red blood cells seem to be preferential targets in this model, as in vivo. Interestingly, endothelial cells from human aorta, umbilical veins or renal glomeruli do not respond to erythropoietin withdrawal in the manner of splenic endothelial cells. This in vitro model continues to yield insights on the mechanisms of neocytolysis.

A rodent model of neocytolysis could greatly facilitate our ability to dissect and experimentally manipulate the process. We have been very successful using AAV—viral vectors to deliver the EPO gene to mice. This establishes stable, high expression of the gene leading to marked polycythemia in the animals. We are co-delivering a tetracycline response control gene and we can successfully turn off the EPO gene expression with tetracycline in in vitro cultured cell lines. We are actively working on turning off erythropoietin with tetracycline in the polycythemic mice, which should precipitate neocytolysis and establish a model for experimental manipulation.

We have established a human model of neocytolysis by injecting volunteers with erythropoietin, increasing their red cell mass, then withdrawing the erythropoietin. We have observed a rapid fall in the number of red cells on erythropoietin withdrawal, just as predicted. One observation that has emerged is that changes in serum ferritin concentration serve as a precise inverse mirror of the changes in red cell mass. Serum ferritin reflects the amount of iron in body stores. As red cell mass increases under the influence of supplemental erythropoietin, serum ferritin falls as iron is mobilized from stores into newly synthesized hemoglobin. When red cell mass falls, as with neocytolysis, ferritin rises rapidly as iron is transferred back to stores. We have found that ferritin very precisely reflects the changes in red cell mass in space, in altitude-

dwellers descending to sea level and now in this erythropoietin-driven human model. The recognition of the utility of ferritin levels in these situations should simplify our ability to study the process. Ferritin levels could also be used clinically as an early measure of the effectiveness of erythropoietin therapy in human disease.

The rate of change of ferritin concentration is much slower when erythropoietin is augmented than when the erythropoietin is decreased which matches our observations in spaceflight that deadaptation to earth's environment as manifest by decrease in red cells occurs quickly. No adverse reaction occurred in normal volunteers receiving erythropoietin for three to six weeks. The model we have established will permit determination of the minimal erythropoietin dose required to prevent deadaptation.

Our discoveries emanating from the unique environment encountered in space have yielded insights on previously unrecognized physiologic and pathophysiologic conditions on earth. We are hopeful that our studies will encourage effective countermeasures for space travelers re-entering a gravitational environment. Our observations clearly impact on such diverse situations as altitude adaptation and de-adaptation, training of elite athletes, anemia of renal disease, optimal erythropoietin dosing schedules, hemolytic anemia and polycythemias. Continuing unraveling of these phenomena will further demonstrate the unforeseen benefits that accrue when basic problems in space are scientifically addressed.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

"Neocytolysis: Mechanisms and Limitations"

2000 ANNUAL PROJECT REPORT

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I. Research Plan Summary

A. Hypotheses, Objectives and Aims from Original Proposal

It has been recognized for decades that astronauts returning from even a few days in space invariably return with a 10-15% decrement in their red cell mass. This "spaceflight anemia" is not benign, but renders the astronaut hypovolemic, orthostatic, "weak" and with decreased aerobic capacity at the mission's most critical juncture, re-entry and landing in a gravitational environment. We believe that the decreased red cell mass, decreased blood volume and altered patterns of blood flow that occur with spaceflight continue to be underestimated as factors in many of the problems experienced by returning astronauts.[1]

We uncovered the physiologic process of "neocytolysis," the selective hemolysis of the youngest circulating red blood cells, in dissecting the cause of spaceflight anemia.[2] Our ferrokinetic studies on SLS-1 and SLS-2 showed that red cell production was normal during the first few days in space, while red cell mass was declining rapidly [3,4]. Red blood cells labeled with ^{51}Cr 12-14 days before launch were found to survive normally. (Actually, the specific activity of ^{51}Cr consistently rose above predicted levels due to a loss of diluting unlabeled cells.) The only possible explanation is that there is selective destruction of a large proportion of circulating red cells which are younger than 12 days old during the first few days in space.

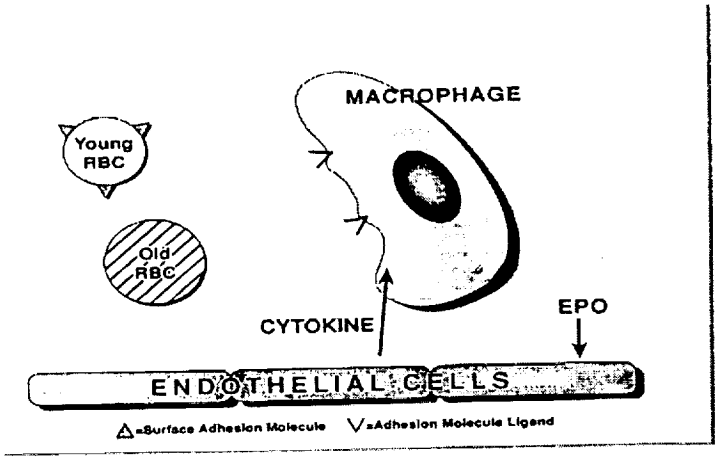


Figure 1 . Model of Neocytolysis.
EPO signals endothelial cells which in turn signal macrophages. This affects their interactions of young red blood cells in the direction of survival versus phagocytic removal.

On entering microgravity, the 20% of blood volume that was held in the extremities immediately pools centrally. The body responds by decreasing plasma volume 15-25% in the first 24 hours. Erythropoietin (EPO) production is shut off in the wake of the central plethora and hemoconcentration.[5] There are many reasons to suspect that it is the fall in EPO below a nadir threshold that initiates neocytolysis, that EPO remains the main regulator of red cell mass in times of excess just as in times of need.[2] Neocytolysis allows rapid physiologic adaptation to the new environment; adaptation would be interminably slow if the traditional EPO-driven red cell production were the only control of red cell mass. Production would fall after EPO had been depressed for a week, and then red cell mass would fall very slowly as senescent cells were not replaced. The fall in

red cell mass and blood volume in space become maladaptive on return to gravity.

If our interpretation of events is correct, then neocytolysis should pertain in situations other than the extraordinary environment of space. One such situation would be in polycythemic acclimated mountain dwellers. On descent to sea level, they would find themselves with too high a red cell mass and blood volume for their new environment. Their EPO production would turn off. This situation is very analogous to astronauts entering microgravity.

To confirm our theory, we studied nine volunteers acclimated to life at 14,500 feet in Cerro de Pasco, Peru. Baseline hematocrits were 55-74%. As predicted, volunteers experienced a 10% decline in red cell mass in the first few days after transport to sea level. EPO levels were profoundly suppressed after descent. Mirroring the rapidity and degree of change of red cell mass were increases in serum ferritin, a strong relationship previously noted in astronauts. Labeling specific aged cohorts of red cells by feeding volunteers ^{13}C-glycine or ^{15}N-glycine revealed survival curves consistent with selective destruction of the youngest circulating red cells. Supporting the role of EPO suppression in initiating neocytolysis, we found that the fall in red cell mass on descent could be totally prevented by the administration of daily low-dose EPO to three volunteers [6,7].

There is pathologic EPO deficiency with renal disease, so our theory predicts that neocytolysis should occur. We withheld EPO therapy from five dialysis patients, and a pattern of transient shortening of red cells survival consistent with neocytolysis emerged in four.[8] Again, blood and stool measurements after ^{13}C-glycine and ^{15}N-glycine administration supported that the youngest red cells had been destroyed. Our finding that neocytolysis contributes to the anemia of renal disease not only establishes the validity of our theory, but it helps to explain previously unanswered questions concerning renal anemia. A hemolytic component of the anemia had been repeatedly demonstrated, never fully explained, but too quickly forgotten with the advent of EPO therapy. Neocytolysis explains the hemolytic component and its elimination by EPO. Neocytolysis also bears on optimal dosing regimens for EPO. It explains the many studies that have found that subcutaneous EPO is more efficient than intravenous. It predicts that daily subcutaneous dosing (to avoid nadirs that precipitate neocytolysis) would be most efficient metabolically and economically.[8]

Neocytolysis should pertain in a number of other situations. One would be "blood-doping" by elite athletes, whether by autologous red cell transfusion or surreptitious EPO administration. Our preliminary data indicate neocytolysis can be induced by this practice (and may underlie some of the adverse events sometimes seen). Hemolytic anemias such as congenital pyruvate kinase deficiency (where young cells are selectively targeted) may involve perturbed neocytolysis. In polycythemia vera, mechanisms must be present to escape

neocytolysis. Thus neocytolysis is a prime example of how discoveries emanating from space exploration can find applicability to better understanding problems on earth.

Much remains unknown about the process of neocytolysis, particularly concerning (1) basic underlying mechanisms and (2) limitations of the process. When first formally proposed, our theory was questioned mainly for the lack of explanation of how the process worked. Our model (Figure 1) presumes that EPO signals endothelial cells which in turn signal reticuloendothelial phagocytes (we suspect mainly in the spleen) and alters the known intimate interaction that occurs with young red blood cells in favor of their destruction. When first proposed three years ago, this model was criticized because endothelial cells were not known to respond to EPO signals and, in fact, EPO was believed to be the most specific of hormones with receptors (and thus effects) limited to early erythroid progenitors. Since then, data from many labs and our own (see later) make the existence of EPO receptors on endothelial cells no longer in question.

The specific aims from our original proposal are to:

1. Establish (for the first time) a panel of assays to define normal red cell adhesion molecule expression, and to determine how they are modified with neocytolysis. We have modified this aim to establish an in vitro model of neocytolysis in which the roles of EPO, adhesion molecules, endothelial cells and reticuloendothelial cells can be dissected.

2. Establish an in vivo model of neocytolysis in humans by giving EPO injections to volunteers and then withdrawing the EPO. This model would allow definition of limitations of the process—how high must red cell mass be raised, how low must EPO be suppressed, for how long, etc. As originally planned, the human model was to be a "testing ground" for the lessons learned about adhesion molecules, and so the development of this model was intentionally delayed until the final year of the project to allow maximal development of informative tools.

3. Establish a rodent model of neocytolysis. Once created, this would greatly facilitate studies of mechanisms, limitations and opportunities for manipulation.

IB. Modifications Required and Rationale for Modifications

With regard to the rodent model of neocytolysis, it was originally proposed that mice would be rendered polycythemic by raising them under hypoxic conditions, then neocytolysis would be precipitated by withdrawing them from those conditions. Originally, we planned to protect some mice from neocytolysis by delivering EPO genes with AAV-vectors accomplishing constitutive EPO expression. We modified our originally proposed rodent model for two reasons.

First, there were substantial problems with the exhypoxic mouse including cage design, mouse viability, reproducibility of effects, etc. Second, our success with encapsulating the murine EPO DNA, modifying the viral vector, establishing excellent in vivo gene expression, and developing technology to co-express a tetracycline-responsive "suicide" gene that could turn off the EPO expression, all suggested a superior rodent model. The model we are now working on is based on creating polycythemia by constitutive expression of the EPO gene and then precipitating neocytolysis by turning off gene expression.

IC. Summary of Progress in Years 1 and 2

IC1. In Vitro Model

We worked under the hypothesis that the spleen might be the site for neocyte destruction because that is where red cells are rapidly destroyed in certain pathological conditions such as anemias, in which red cell phagocytosis by endothelial cells and subjacent macrophages is observed. It is well known that young red blood cells in particular must intimately interact with splenic macrophages. The young red cells are pitted and culled of Howell-Jolly bodies, Pappenheimer bodies and other inclusions and their surfaces are modeled with smoothing of pits and removal of excess lipids. Therefore, we isolated and characterized human splenic endothelial cells (HSEC). These endothelial cells, unlike those from other sources such as the aorta, glomerulus, and umbilical vein, responded to erythropoietin deprivation by increasing their permeability to a sugar molecule. However, we were unable to measure erythropoietin receptors on the splenic endothelium by radioimmunoassay, even though endothelial cells such as those from the brain and umbilical vein had been reported to possess such receptors.

We also investigated possible ways to physically separate young and old red cells to compare their interactions with endothelial cells and macrophages, the other element of an in vitro model system. We characterized the adhesion molecule expression of the red cells, both as a way of checking the validity of the separation (since young and old red cells differ in their level of expression of several adhesions) and as a prelude to investigating the role of adhesion molecules in the interaction of red cells with the other elements of the system. We used several approaches to separating young versus old cells, including those based on density (phthalate ester gradients) and volume (the amount of a vital dye incorporated into the cells). Although highly efficacious in separating young and old cells, both of these approaches resulted in damage to the red cells and indiscriminate high-level uptake by monocyte-derived macrophages without opsonization. Therefore, a more benign way to separate the cells was sought.

IC2. Animal Model

As outlined and graphed in figures 1 and 2 of our report last year, Dr. Byrne has been remarkably successful in transferring the EPO gene to mice using AAV vectors. Dose-response curves relating AAV dose to both serum EPO levels and to hematocrit demonstrated stable, long-term physiologically active gene expression. At higher doses, animals maintained hematocrits above 80% for a year. Studies had begun to co-express a tetracycline response element with the EPO gene.

II. Progress, results, and accomplishments.

A. Erythropoietin receptors on splenic endothelial cells.
Although we had shown a physiological response to EPO withdrawal by HSEC, we had not been able to show the presence of the receptor as measured by radioimmunoassay. Therefore, we changed techniques, immunoprecipitating the receptor with a monoclonal antibody, followed by electrophoresis and immunoblotting with a specific antiserum. The EPO receptor from splenic endothelium had an identical molecular weight to that from K562 erythroleukemia cells used as a positive control (Fig. 2). The receptor was present only when the endothelial cells had been grown in the presence of physiological amounts of EPO, indicating that the receptors can be regulated on these cells.

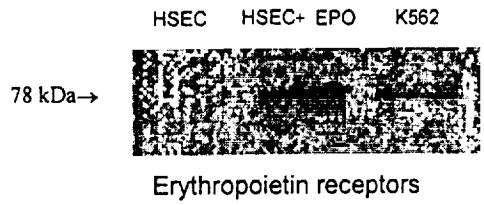


Fig. 2. PO receptors on human splenic endothelial cells grown without PO or with 20 mU/ml PO. K562 cells were used as a positive control.

B. Morphological changes in response to EPO deprivation.
Since HSEC had responded to EPO withdrawal by increasing their permeability, it was of interest to document any morphological changes that might accompany this response. Scanning electron microscopy (SEM) was performed on HSEC that had been exposed to no EPO throughout the culture period (-/-), to 20 mU/ml throughout (+/+), or to 20 mU/ml for 72 hours followed by 24 hours of deprivation (+/-). SEM of both the -/- and +/+ cultures

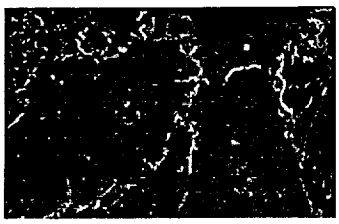


Fig. 3A. SEM of -/- HSEC.



Fig3B SEM of -/- HSEC.

indicate that these cells grow as a flat confluent monolayer with relatively few microvilli and with membrane ridges along the cell borders (Fig. 3, A and B). The +/- cultures, on the other hand, have relatively simple borders and many microvilli distributed over the cell surfaces (Fig. 3C). These morphological changes may lead to the observed permeability changes in the +/- cultures by means of increased transport through microvilli or a change in the structure of cell borders resulting in an altered barrier function.

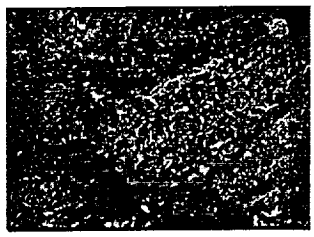


Fig. 3C. SEM of +/- HSEC.

C. Separation and characterization of red cells and their association with endothelial cells and macrophages.

Since the hypothesis for the in vitro model of neocytolysis was that endothelial cells deprived of EPO should either interact preferentially with young red cells or should promote such an interaction between associated macrophages and neocytes, a reliable method for obtaining young red cells was required. Since previously used methods had damaged the red cells, we separated red cells by a cell sorter based on their size as measured by forward-angle light scatter. Spherocytosis was induced by hypotonic buffer treatment, and the smallest and largest 20% of the cells were sorted (Fig. 4).

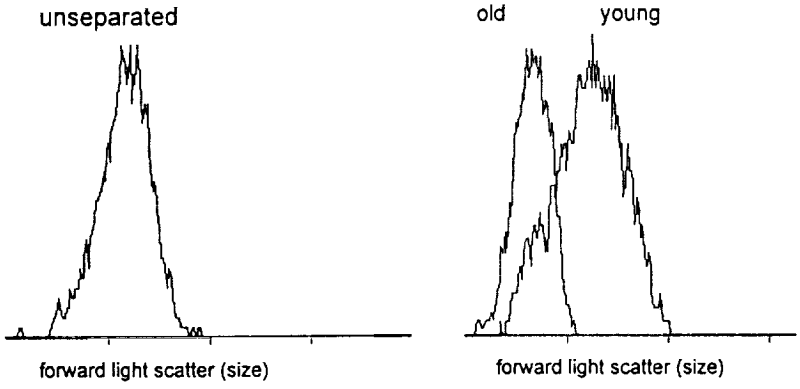


Fig. 4. RBC size separation. Analysis of the starting population is on the left, and analysis of the separated cells is on the right.

To bring together all the elements of an <u>in vitro</u> model, we had to include macrophages as well as the splenic endothelial cells and red cells. Therefore, we obtained monocyte-derived macrophages (MDMs) by allowing blood mononuclear cells to adhere to plastic for 24-48 hours. These cells were then scraped from the plastic and allowed to adhere to collagen pads containing 20 µg/ml human fibronectin. Endothelial cells were plated on top of the adherent MDMs and treated according to the usual cycle of 72 hours with and 24 hours

without EPO. Control groups were left untreated or treated with EPO throughout. Red cells were then sorted into young and old populations and added to the cultures at 10^6 per well. After a further overnight culture, nonadherent red cells were removed by vigorous washing. All the remaining cells were lysed by detergent, and the hemoglobin content analyzed by spectrophotometry at 540 nm.

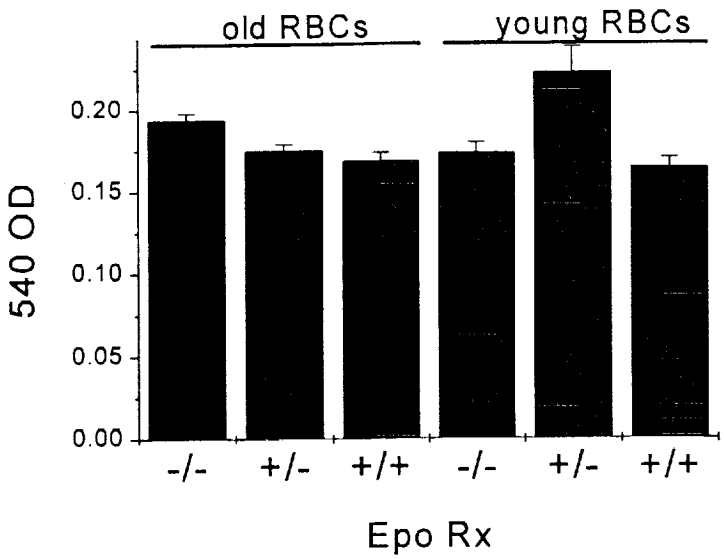


Fig. 5. Hemoglobin content of cultures treated with and without PO and exposed to young or old red cells.

There was little difference among cultures with respect to their interaction with young or old red cells, except for EPO-deprived cultures (+/-) capturing young red cells (Fig. 5). This capture required the presence of both the endothelial cells and macrophages, since cultures of either cell type alone treated in the same manner showed no preferential uptake of red cells (Fig.6). Phase microscopy of the cultures before lysis revealed that red cells were contained within macrophage processes below the endothelial cells in the collagen pad. This interaction took place despite the fact that the endothelial cells were confluent over the macrophages as determined by silver staining. Indeed, if the endothelial cells did not reach confluence during the four-day culture period, no preferential interaction with the young red cells in the EPO-deprived cultures took place (data not shown). Thus, upon EPO deprivation, signaling between the endothelium and subjacent macrophages may take place to allow passage of young red cells through the endothelium and uptake by the macrophages.

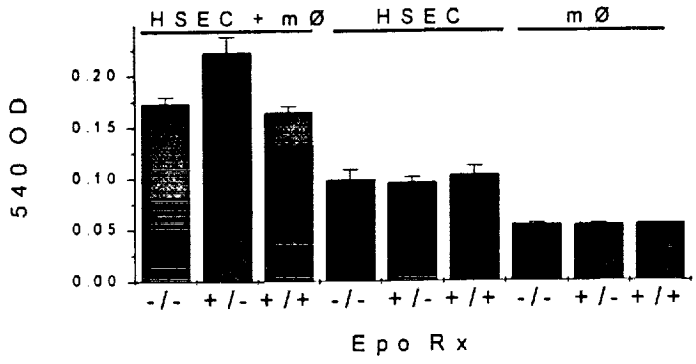


Fig. 6. Hemoglobin content of mixed and single cultures.

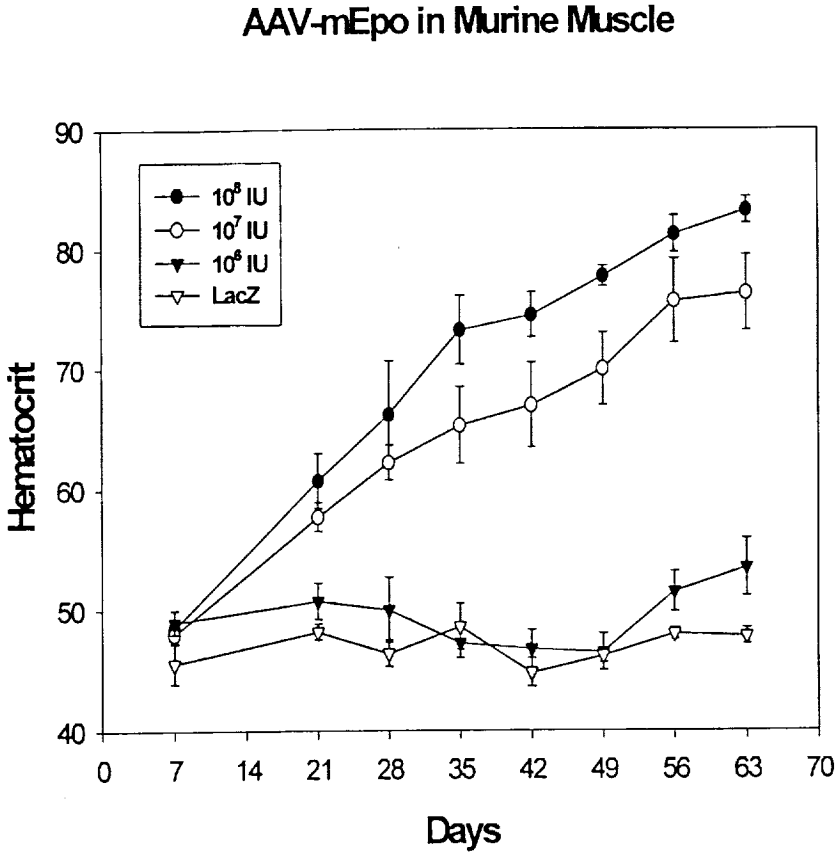


Figure 7

IID. Progress of Animal Model

In our current experiments, AAV vectors have co-delivered murine EPO DNA along with a tetracycline responsive control element to C57B6 mice. As shown in Figure 7, there continues to be physiologic dose-responsive expression of EPO.

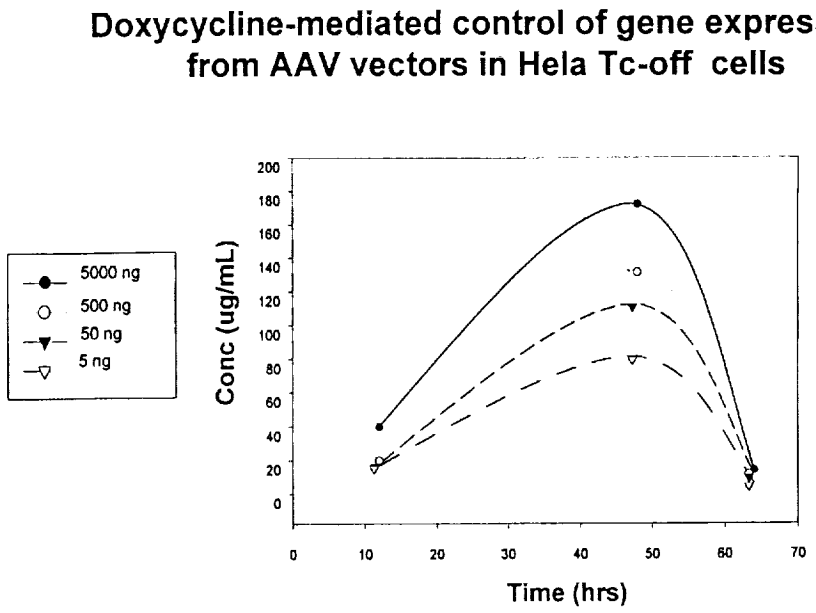


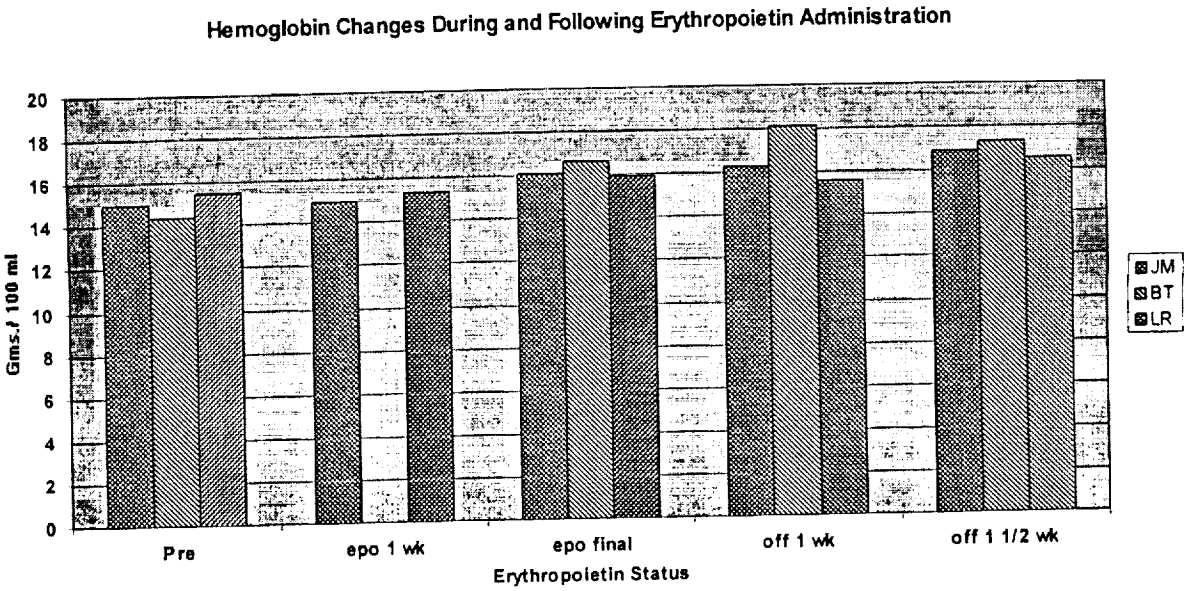
Figure 8

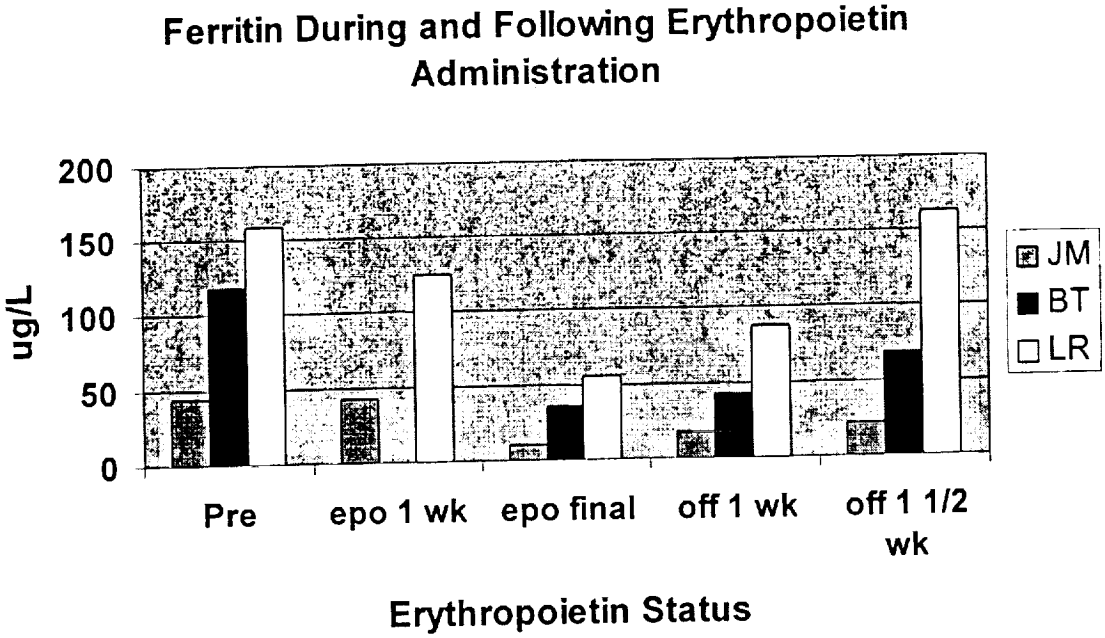
Figure 8 shows that in an in vitro model using Hela Tc-off cells, tetracycline can modulate and turn off EPO expression. At present, we are working on turning off EPO secretion with tetracycline in the living mouse.

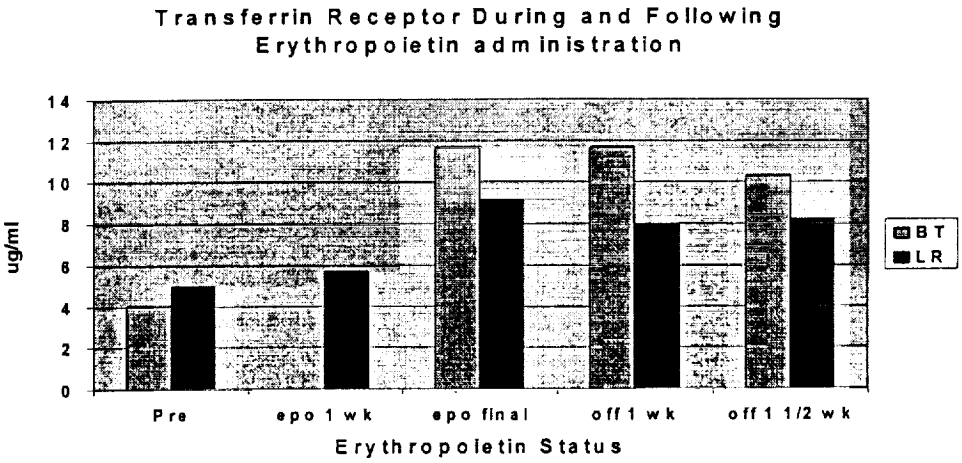
IIE. Human Model

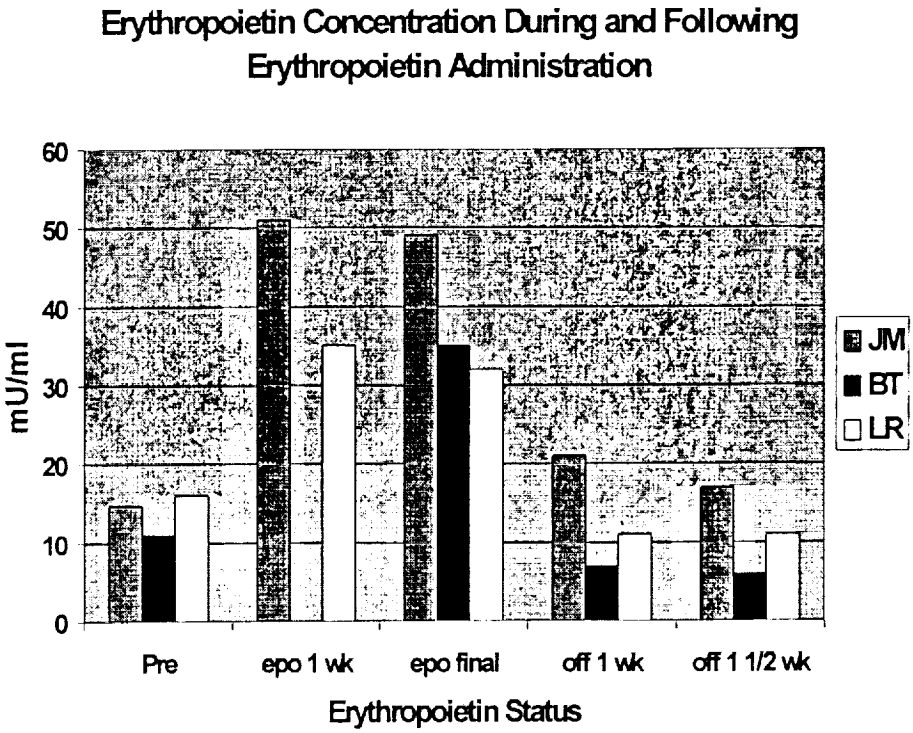
We have completed studies on three human volunteers. All received daily subcutaneous EPO to induce erythrocytosis. EPO was then discontinued to precipitate neocytolysis.

Following are the changes in red cell mass and hemoglobin were precisely correlated with changes in serum ferritin. The ferritin fell during EPO administration as storage iron was mobilized into new red blood cells, then ferritin rose very rapidly after EPO withdrawal reflecting neocytolysis. Changes observed in different populations of reticulocytes, those with high RNA content and those with lower concentrations of RNA.

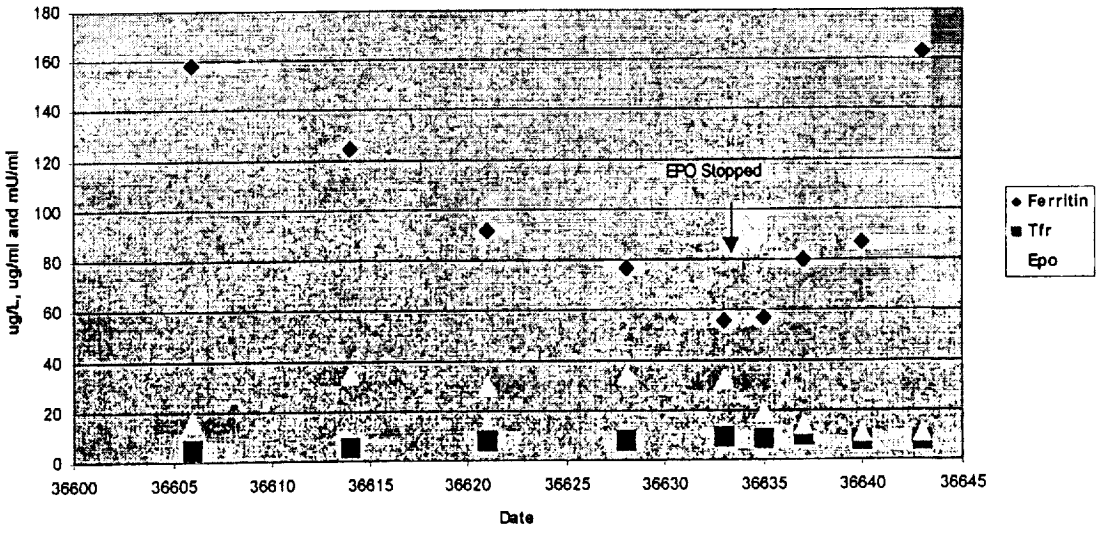

Hemoglobin Changes During and Following Erythropoietin Administration
Gms./ 100 ml
20
18
16
14
12
10
8
6
4
2
0
Pre
epo 1 wk
epo final
off 1 wk
off 1 1/2 wk
Erythropoietin Status
JM
BT
LR


Ferritin During and Following Erythropoietin Administration
ug/L
200
150
100
50
0
Pre
epo 1 wk
epo final
off 1 wk
off 1 1/2 wk
Erythropoietin Status
JM
BT
LR


Transferrin Receptor During and Following
Erythropoietin administration
ug/ml
14
12
10
8
6
4
2
0
Pre
epo 1 wk
epo final
off 1 wk
off 1 1/2 wk
Erythropoietin Status
BT
LR


Erythropoietin Concentration During and Following
Erythropoietin Administration
mU/ml
60
50
40
30
20
10
0
Pre
epo 1 wk
epo final
off 1 wk
off 1 1/2 wk
Erythropoietin Status
JM
BT
LR

Serum Ferritin, Transferrin Recetor and Erythropoietin during and after Erythropoietin Administration



The changes in serum erythropoietin, serum ferritin and transfusion receptor which were observed in each of the three volunteers. Serum ferritin decreased gradually during the period of EPO administration. Erythropoietin increased from 10-12 m U/ml to 35-40 mU/ml and levels for transferrin receptors nearly doubled. When erythropoietin injections were discontinued serum ferritin increased abruptly reaching pretreatment levels in less than one week. Transferrin receptor levels decreased over three to four days and erythropoetin levels decreased below baseline values after 2-3 days.

IIF. Complementary Studies

In the introduction, we referred to our studies in the Peruvian Andes which helped to confirm the existence of neocytolysis. Within the last year, we have been able to fully analyze the data obtained and to prepare a manuscript for publication.(7) We have recently acquired funding to continue studies of the roles of EPO and neocytolysis in adaptation to and from high altitude environments in Peru.

In the last year, we have published studies showing that neocytolysis contributes to the anemia of renal disease (8). Neocytolysis explains previously unanswered problems in renal anemia, such as the never fully explained hemolytic component. Neocytolysis predicts that formerly standard EPO dosing regimens using three intravenous boluses weekly would be very inefficient because the EPO peak would effect erythroid

progenitor proliferation but this would be followed by nadir levels precipitating neocytolysis. We are currently comparing the degree of neocytolysis seen in dialysis patients on intravenous EPO compared to subcutaneous EPO.

Neocytolysis should be precipitated in elite athletes who take autologous blood transfusions or surreptitious EPO in an effort to enhance performance. Our studies have attracted interest from Olympic trainers. We have recently participated in studies of red cell changes occurring in Olympic hopefuls training at high altitude. Their increase in red cell mass is quickly lost due to neocytolysis on descent figure. We are investigating whether reliable markers of neocytolysis can be used to help identify athletes who disregard prohibitions against "blood doping."

III. Implications for future research

The in vitro model system described above may be useful for elucidating the mechanisms involved in preferential destruction of young red cells under conditions of EPO withdrawal. For example, do adhesion molecules facilitate the recognition of neocytes by either endothelial cells or their associated macrophages which we have confirmed is expressed at higher levels on young versus old red cells. If signal transduction between endothelial cells and macrophages takes place after EPO withdrawal, then agents blocking specific pathways may define the means of communication between the two cell types. Morphological studies are planned to identify the means of red cell passage through the endothelium and into macrophages. For example, do macrophage processes extend between endothelial cells to contact red cells directly, or do the red cells contact the endothelial cells first. What is the means of passage of the red cells past the endothelium? Definition of the role of each cell type may allow intervention and control of the process.

We are currently exploring limitations of neocytolysis in the human model. The availabilities of both the human and animal models should prove useful in the future to further dissect the process of neocytolysis, determine the adhesion molecule targets and to attempt to manipulate the process. Our in vivo studies indicate that a mild plethora can be induced in normal volunteers after two to three weeks of erythropoietin injections. When the injections are stopped erythropoietin levels rapidly increase mirroring neocytolysis as occurs in astronauts entering microgravity or residents of high altitude who come to sea level. This in vivo model will afford the opportunity to determine the minimum dose of erythropoietin required to prevent neocytolysis. After establishing the minimal dose required, this dose could be given to astronauts with the expectation that neocytolysis can be prevented and that the red cell mass will not fall during short term spaceflight. We believe that the maintenance of a red cell mass optimal for earth's gravity will reduce the magnitude of orthostatic hypotension following return to earth.

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10. Trial J, Rice L, Alfrey CP. Erythropoietin withdrawal alters interactions between young red cells, splenic endothelial cells, and macrophages. An in vitro model of neocytolysis. Submitted, J Invest. Med.

NSBRI RESEARCH PROGRAM
MUSCLE ALTERATIONS AND ATROPHY

Team Leaders: **Hamilton, S. L.** **Baylor**
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Schwartz, R. J.	**PI**	**Baylor**	**GH/IGF-I Transgene Expression on Muscle Homeostasis**
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Demayo, F. J.	CO-I	Baylor	
O'Malley, B. W. Jr.	CO-I	Hopkins/SOM	
Goldberg, A. L.	**PI**	**Harvard**	**Pharmacological Inhibitors of the Proteosome in Atrophying Muscles**
Baldwin, K. M.	CO-I	UC, Irvine	
Epstein, H. F.	**PI**	**Baylor**	**Molecular Signaling in Muscle Plasticity**
Booth, F. W.	CO-I	University of Missouri - Columbia	
Schwartz, R. J.	CO-I	Baylor	
Hamilton, S. L.	**PI**	**Baylor**	**Activity Dependent Signal Transduction in Skeletal Muscle**
Armstrong, R. B.	CO-I	Texas A&M	
Reid, M. B.	CO-I	Baylor	
Rosenthal, N. A.	**PI**	**Harvard**	**Molecular Mechanisms Regulating Muscle Fiber Composition Under Microgravity**
Baldwin, K. M.	CO-I	UC, Irvine	
Mosier, D. R.	**PI**	**Baylor**	**Motoneuron Influences on Muscle Atrophy in Simulated Microgravity Induced Muscle Atrophy**
Appel, S. H.	CO-I	Baylor	
Booth, F. W.	CO-I	University of Missouri - Columbia	
Schwartz, R. J.	CO-I	Baylor	

Muscle Alterations and Atrophy Program Report is not included

Hamilton, S.L.
Schwartz, R.J.

National Space Biomedical Research Institute

ANNUAL PROJECT REPORT

Project Title: Role of the GH/IGF-I Axis, Loading, and Exercise on Muscle Mass Homeostasis

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Robert J. Schwartz, Principal Investiogator

Date

EXECUTIVE SUMMARY

We propose to test the hypothesis that the growth hormone/ insulin like growth factor-I axis through autocrine/paracrine mechanisms may provide long term muscle homeostasis under conditions of prolonged weightlessness. As a key alternative to hormone replacement therapy, ectopic production of hGH, growth hormone releasing hormone (GHRH), and IGF-I will be studied for its potential on muscle mass impact in transgenic mice under simulated microgravity. Expression of either hGH or IGF-I would provide a chronic source of a growth-promoting protein whose biosynthesis or secretion is shut down in space. Muscle expression of the IGF-I transgene has demonstrated about a 20% increase in hind limb muscle mass over control nontransgenic litter mates. These recent experiments, also establish the utility of hind-limb suspension in mice as a workable model to study atrophy in weight bearing muscles. Thus, transgenic mice will be used in hind-limb suspension models to determine the role of GH/IGF-I on maintenance of muscle mass and whether concentric exercises might act in synergy with hormone treatment. As a means to engineer and ensure long-term protein production that would be workable in humans, gene therapy technology will be used by to monitor muscle mass preservation during hind-limb suspension, after direct intramuscular injection of a genetically engineered muscle-specific vector expressing GHRH. Effects of this gene-based therapy will be assessed in both fast twitch (medial gastrocnemius) and slow twitch muscle (soleus). End-points include muscle size, ultrastructure, fiber type, and contractile function, in normal animals, hind limb suspension, and reambulation.

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RESEARCH PLAN SUMMARY

A. OBJECTIVES & SPECIFIC AIMS

A current goal of the National Space Biomedical Research Institute is to develop countermeasures that allow humans to live and work in microgravity for duration over a year and to minimize readapting to Earth's gravity, and optimize crew safety, well-being, and performance. We propose to test the hypothesis that the GH/IGF-I axis through autocrine/paracrine mechanisms may provide long term muscle homeostasis under conditions of prolonged weightlessness. As a key alternative to hormone replacement therapy, ectopic production of hGH, growth hormone releasing hormone (GHRH), and IGF-I will be studied for its potential on muscle mass impact in transgenic mice under simulated microgravity. Transgenic mice will be used in hind-limb suspension models to determine the role of GH/IGF-I on maintenance of muscle mass and whether concentric exercises might act in synergy with hormone treatment. As a means to engineer and ensure long-term protein production that would be workable in humans, gene therapy technology will be used by to monitor muscle mass preservation during hind-limb suspension, after direct intramuscular injection of a genetically engineered muscle-specific vector expressing GHRH. Effects of this gene-based therapy will be assessed in both fast twitch (medial gastrocnemius) and slow twitch muscle (soleus). End-points include muscle size, ultrastructure, fiber type, and contractile function, in normal animals, hind limb suspension, and reambulation. The following aims are:

Aim I: Does IGF-I and the IGFI receptor provide signaling for muscle mass homeostasis in response to unloading, overloading and exercise?

Aim II: Does exercise and GH/IGF-I axis synergize in alleviating muscle atrophy? To determine the role of overexpression of hGH, and IGF-I in transgenic mice on muscle mass accretion under condition of hindlimb underloading?

Aim III Does increased levels of GHRH potentiate the production of IGF-I in underloaded muscle?

Answering these aims will provide information that could: a) refine training programs for astronauts, b) provide the basis for the development of pharmaceutical countermeasures, and c) provide marker(s) to assess the success of exercise countermeasures in the prevention of muscle atrophy in astronauts.

Specific Hypotheses

1. GHRH/GH/IGF-I axis through autocrine and paracrine mechanisms will provide long term muscle hypertrophy under conditions of prolonged weightlessness or muscle unweighting due to suspension.

2. Locally produced IGF-I would be effective in preventing unloading-induced atrophy of skeletal muscle

3. In tandem with GHRH/GH/IGF-I axis synergy with resistive exercise regimens will block muscle atrophy under microgravity. In this proposal.

4. Enhancement in muscle mass could occur by GHRH secretagogue activity increasing the systemic levels of growth hormone, which then elicits increased level of systemic or local IGF-I production in muscle

1) **Role of IGF-I as a countermeasure:** Previously Dr. Richard Grindeland has shown that a combination of IGF-I administraion and ladder climbing was more effective than either alone in attenuating atrophy of unloaded skeletal muscles in hypophysectomized rats. These animals without pituitary glands would have no growth hormone or glucocorticoid hormones. Since astronauts have pituitary glands, it is important to confirm these findings in animals with intact pituitary glands. The effects seen by these investigators may have been a result of using hypophysectomized rats. We hypothesized that a combination of IGF-I overexpression and stretching exercise would be more effective than either alone in attenuating muscle atrophy from unloading in unloaded muscles of hindlimbs of mice with intact pituitaries.

We tested localized IGF-I transgene expression in underloaded muscle by using hind limb suspension model. Twenty male transgenic mice (about 6-mo-old) harboring the human IGF-I gene driven by regulatory regions from the chicken skeletal alpha actin promoter extending to -424 bp upstream of the transcription initiation start site, the first intron, and the 3' untranslated region (SK733IGF-I3'SK) were used to asses the potential of locally produced IGF-I to prevent unloading induced atrophy. In our recent study with Dr. Frank Booth, we observed that the local over expression of IGF-I was associated with a higher absolute muscle mass in the weight bearing, transgenic mice (GAST:+21%,TA:+15%) compared to the FVB, nontransgenic mice, even though tibial lengths differed by only 2.7%. Following 14 days of suspension, however, the percentage loss of mass in the GAST and TA of the transgenic mice(about 20%) did not differ from the HU-induced atrophy in the FVB wild type mice, resulting in an absolute mass of these two muscles in the HU transgenics equivalent to that of weight bearing FVB mice. We also found that endogenous IGF-I mRNA level was unaltered in atrophying GAST muscle of nontransgenic mice, suggesting that a down regulation of IGF-I may not be involved in unloading-induced skeletal muscle atrophy as we had hypothesized.

Conversely, we reported that the expression of the skeletal α-actin driven IGF-I transgene is down regulated following 14 days of HU and that this expression also occurs in a fiber type-specific manner. Yet, despite this down regulation, the GAST and TA of the transgenic mice still expressed hIGF-ImRNA and peptide levels that were magnitudes higher than the corresponding muscle in the nontransgenic FVB mice. Therefore, our hypothesis that locally produced IGF-I would be more effective in preventing unloading-induced atrophy of skeletal muscle was not supported. We also conclude that elevated IGF-I expression alone is ineffective in preventing

unloading-induced muscle atrophy within fast-twitch skeletal muscle. We observed no facilitative interaction by IGF-I for the attenuation of unloading atrophy by stretching unloaded muscles (See Criswell, D.A., F.W. Booth, F. DeMayo, R.J. Schwartz, S.E. Gordon, and M.L. Fiorotto. Over-expression of IGF-I in skeletal muscle of transgenic mice does not prevent unloading-induced atrophy. *Am. J. Physiol.* 275:E373-E379, 1998)

One of the key factors responsible for the age-associated reduction in muscle mass may be that satellite cell proliferation potential (number of doublings contained within each cell) could become rate limiting to old muscle regrowth. No studies have tested whether repeated cycles of atrophy-regrowth in aged animals deplete the remaining capacity of satellite cells to replicate or what measures can be taken to prevent this from happening. We hypothesized that there would be a pronounced loss of satellite cell proliferative potential in gastrocnemius muscles of aged rats (25- to 30-mo-old FBN rats) subjected to three cycles of atrophy by hindlimb immobilization (plaster casts) with intervening recovery periods. Our results indicated that there was a significant loss in gastrocnemius muscle mass and in the proliferative potential of the resident satellite cells after just one bout of immobilization. Neither the muscle mass nor the satellite cell proliferation potential recovered from their atrophied values after either the first 3-wk or later 9-wk recovery period. Remarkably, application of insulin-like growth factor I onto the atrophied gastrocnemius muscle for an additional 2 wk after this 9-wk recovery period rescued approximately 46% of the lost muscle mass and dramatically increased proliferation potential of the satellite cells from this muscle.

Interest is growing in methods to extend replicative life span of non-immortalized stem cells. Most recently, our study (See Chakravarthy MV, et al 2000 IGF-I extends in vitro replicative life span of skeletal muscle satellite cells by enhancing G1/S cell cycle progression via the activation of PI3'-kinase/Akt signaling pathway. J. Biol. Chem In Press) has provided new insights into the role of IGF-I and muscle repair. Using the IGF-I transgenic mouse in which the IGF-I transgene is expressed during skeletal muscle development and maturation prior to isolation and during culture of satellite cells, as a model system, we elucidated the underlying molecular mechanisms of IGF-I-mediated enhancement of proliferative potential of these cells. Satellite cells from IGF-I transgenic muscles achieved at least five additional population doublings above the maximum that was attained by wildtype satellite cells. This IGF-I-induced increase in proliferative potential was mediated via activation of the PI3'-kinase/Akt pathway, independent of MAP-kinase activity, facilitating G1/S cell cycle progression via a downregulation of p27KIP1. Adenoviral-mediated ectopic overexpression of p27KIP1 in exponentially growing IGF-I transgenic satellite cells reversed the increase in cyclin E-cdk2 kinase activity, pRb phosphorylation, and cyclin A protein abundance, thereby implicating an important role for p27KIP1 in promoting satellite cell senescence. These observations provide a more complete dissection of molecular events by which increased local expression of a growth factor in mature skeletal muscle fibers extends replicative life span of primary stem cells than previously known. These studies show that the mode of presentation of growth factors to atrophying skeletal muscle is important.

In addition. Bert O' Malley Jr. a coinvestigator, has investigated the potential for using myogenic vectors driving IGF-I for muscle repair. In a recent report (Shiotani A, O'Malley BW Jr, Coleman ME, Alila HW, Flint PW (1998) Reinnervation of motor endplates and increased muscle fiber size after human insulin-like growth factor I gene transfer into the paralyzed larynx. Hum Gene Ther. 1998 ;9(14):2039-47). A muscle-specific nonviral vector containing the alpha-actin promoter and hIGF1 gene formulated with polyvinyl polymers was injected into denervated adult rat thyroarytenoid muscle. The effects on animals given a single injection (n = 16) vs those given multiple injections (n = 14) vs control groups (n =18) were evaluated. Twenty-eight days after the first injection, gene expression, muscle fiber size, motor endplate length, and nerve-to-motor endplate contact were evaluated. Gene expression, detected by reverse transcriptase polymerase chain reaction for hIGF1 messenger RNA, occurred in 13 (81%) of 16 animals receiving single injections and 14 (100%) of 14 animals receiving multiple injections. Compared with controls, hIGF1-transfected animals in both single- and multiple-injection groups had a significant increase in the lesser diameter of muscle fiber, a significant decrease in motor endplate length, and a significant increase in the percentage of endplates with nerve contact ($P < .05$ for all). There was no statistical difference between single- and multiple-injection groups. Applied to laryngeal paralysis, hIGF1 gene therapy provides an opportunity to augment surgical treatment modalities by the prevention or reversal of muscle atrophy, and enhancement of nerve sprouting and muscle reinnervation. Although the percentage of denervated muscles demonstrating hIGF1 expression was increased following multiple injections, no difference was observed in the biological response compared with that in the single-injection treatment groups. Further investigation will be conducted to assess long-term benefits and physiological responses and to define the limitations of this potentially valuable therapeutic strategy.

Role of Growth Hormone Releasing Hormone Gene Therapy as Counter Measure: Regulated expression of the growth hormone/insulin-like growth factor axis is essential for optimal linear growth, as well as homeostasis of carbohydrate, protein, and fat metabolism. GH synthesis and secretion from the anterior pituitary is stimulated by the natural GH secretagogue growth hormone releasing hormone (GHRH) and inhibited by somatostatin, both hypothalamic hormones. GH increases production of insulin-like growth factor I (IGF-I), primarily in the liver, and possibly other target organs. Increased levels of serum IGF-I, in turn, feeds back on the hypothalamus and anterior pituitary to inhibit GHRH release and GH secretion. The pulsaile pattern is thought to arise from alternating episodes of stimulation by GHRH and inhibition by somatostatin. The endogenous rhythm of GH secretion becomes entrained to the imposed rhythm of exogenous GH administration. It is well established that ectopically secreted GHRH, as mature peptide or truncated molecules (as seen with pancreatic islet cell tumors and various located carcinoids) are often biologically active and can even produce acromegaly.

Administration of recombinant GHRH to GH-deficient children or adult humans augment IGF-1 levels, increases GH pulsatile secretion proportionally to GHRH dose, with preserve response to bolus doses of GHRH. Thus, the GHRH administration represent a more physiological alternative of increasing subnormal GH and IGF-1 levels. Most importantly,

however, is that GH secretion is effected at vanishingly low levels of GHRH (10 pg/ml) in the blood supply. Thus, by employing a gene therapy approach, the human GHRH cDNA could be targeted into peripheral organs and expressed by the transfected cells and the peptide processed, secreted, transported to the anterior pituitary, where it could stimulate GH release.

To establish the baseline expression of the human GHRH cDNA, driven by skeletal actin promoter in a DNA plasmid after in vivo administration directly into mouse muscle. These experiments will create the foundation for demonstrating reproducibility, dose response, and duration of expressed product; persistence and state of DNA; and, initial safety. The route and method of vector DNA administration to muscle are critical steps towards controlling the expression of a therapeutic product in somatic gene therapy. Conventional intramuscular injections have been shown to be an effective means of introducing genes into muscle and achieving detectable levels of gene expression. Studies have shown that DNA introduced into expression from muscle has been reported to persist for several months to even up to a year in vivo. In a gene therapy approach, the human GHRH cDNA could be targeted into peripheral organs and expressed by the transfected cells and the peptide processed, secreted, transported to the anterior pituitary, where it could stimulate GH release. Skeletal muscle can be transfected in vivo by direct plasmid DNA injection, which can be expressed at significant levels for different periods of time, up to 19 month. A 228bp fragment of hGHRH, which encode for the 31 amino acid signal peptide and the entire mature peptide hGHRH(1-44)OH (Tyr1-Leu44), was cloned into a skeletal muscle actin promoter followed by the 3' untranslated region of human growth hormone cDNA.

Muscle gene therapy, synthetic muscle promoters: Skeletal muscle is an attractive target for somatic gene therapy because of its long life span, ready accessibility for intramuscular injections, large capacity for protein synthesis and secretion. Moreover, muscle tissue is highly vascularized and has a high rate of blood flow; thus, allowing de novo proteins to readily enter the systemic circulation. Importantly, direct administration of soluble or precipitated plasmid DNA into muscle leads to expression of recombinant proteins in muscle cells. Delivery of plasmid DNA can persist in an episomal state directing the expression of recombinat proteins for months to years. However, the limiting problem in using muscle as a gene therapy target has been the relatively low levels of expression that has been achieved with muscle specific vectors. Recently, we described the stratergy and characterization of novel muscle synthetic promoters, whose transcriptional potency in terminally differentiated muscle greatly exceeds that of the natural myogenic skeletal α-actin gene promoter and viral promoters.

Analysis of the organization of several strong muscle promoters and enhancers, with regards to groupings of cis-acting regulatory elements and their interactions with myogenic regulatory factors led us to formulate a simple stratergy to constuct synthetic muscle promoters. Typically, myogenic restricted gene promoters, such as the α-actins, display complex organization usually involving combinatorial interactions of several myogenic transfactors through pairs of specific regulatory elements. These elements which are evolutionary conserved and primarily responsible for tissue-specific expression in adult skeletal muscle, influenced our

decision in selecting four core myogenic regulatory elements for generating synthetic promoters. By random assembly of E-box, Mef-2, TEF-1 and SRE sites into synthetic promoter recombinant libraries, and screening hundreds of the resultant clones for transcriptional activity, a few artificial promoters were discovered whose transcriptional potency greatly exceeds that of natural myogenic and viral gene promoters.

In vitro activity of synthetic promoters. As a screening method for the strength of the SP, we measured the *in vitro* luciferase activity of more then 1000 different clones in 96 well dishes in transiently transfected chicken primary myoblasts. As a muscle specific expression control, we used a 448bp fragment (-424 / +24) of the avian skeletal a-actin gene, active in differentiated skeletal muscle cells, but not in myoblasts. Cytomegalovirus (CMV) basic promoter was used as an ubiquitous promoter control. SP and SK448 transfected cells were placed into differentiation media for 72 hours to initiate withdrawal from the cell cycle and to induce post-fusion differentiation and muscle-specific promoter activation; at the end of this period the cells were harvest and assayed for the reporter gene.

One of the most active constructs, SPc5-12, was then tested over a 96 hour time-course to monitor for the activation of this synthetic promoter, in comparison to CMV and SK actin promoters, during primary avian muscle cell myogenesis in culture where replicating myoblasts withdraw from the cell cycle, fuse and form multinucleated terminally differentiated myotubes (figure 2B). The CMV promoter was active in both myoblasts and myotubes at similar levels (1.05±0.06 relative units (RU)/mg protein at 24h, 1.22±0.22 RU/ mg protein at 96h). The SK448 expression increased only after 48 hours (0.17±0.016 RU/ mg protein at 48h, 0.37±0.09 RU/ mg protein at 72h, 0.41±0.06 RU/ mg protein at 96h), which correspond to the pattern of activation of SK- a-actin promoters, active in myotubes but not in replicating myoblasts. SPc5-12 was not active at 24h and mimicked the pattern of activation of SK448, but it was 10 fold more active than SK448 and 2-6 fold higher then CMV promoter at 96h (2.27±0.23 RU/ mg protein at 48h, 3.62±0.91 RU/ mg protein at 72h and 7.25±0.48 RU/ mg protein at 96h).

Growth hormone releasing hormone gene therapy in large mammals, testing of an early stage countermeasure: Although GHRH protein therapy entrains and stimulates normal cyclical GH secretion with virtually no side effects; the short half-life of GHRH in vivo, requires frequent (one to three times a day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administration. Thus, as a chronic treatment, GHRH administration is not practical. However, extracranial secreted GHRH, as processed protein species (Tyr1-40 or Tyr1-Leu44) or even as shorter truncated molecules are biologically active. Importantly, a low level of GHRH (100 pg/ml) in the blood supply stimulates GH secretion and makes GHRH an excellent candidate for gene therapeutic expression. Direct plasmid DNA gene transfer is currently the basis of many emerging gene therapy strategies, which does not require viral genes or lipid particles. Skeletal muscle is a preferred target tissue, because muscle fiber has a long life span and can be transduced by circular DNA plasmids that express over months or years in an immunocompetent host. Previously, we reported that human GHRH cDNA could be delivered to muscle, by an injectable myogenic expression vector in mice, where it transiently stimulated GH secretion

barely over a period of two weeks. We have now optimized this injectable myogenic vector system by incorporating a powerful synthetic muscle promoter coupled with a novel protease resistant GHRH molecule (pSP-GHRH-HV), possessing substantially longer half-life and greater GH secretory activity, together with improved muscle delivery via highly efficient electropration technology. We wanted to test this GHRH gene therapy in young pigs because it would allow us to evalate it's efficacy in a large mammal with skin and muscle characteristics more similar to humans.

Intramuscular injection of plasmid DNA in pigs: Three groups of five, 3-4 weeks old hybrid cross barrows (Yorkshire, Landrace, Hampshire and Duroc), were used in the GHRH studies. The animals were individually housed with ad lib access to water, and 6% of their body weight diet (24% protein pig meal, Producers Cooperative Association, Bryan, TX). The animals were weighted every other day, at 8:30 am, and the feed was subsequently added. Animals were maintained in accordance with NIH Guide, USDA and Animal Welfare Act guidelines.

The plasmid pSPc5-12 contains a 360bp SacI/BamHI fragment of the SPc5-12 synthetic promoter [23] in the SacI/BamHI sites of pSK-GHRH backbone. The wild type and mutated porcine GHRH cDNAs were obtained by site directed mutagenesis of human GHRH cDNA (Altered Sites II in vitro Mutagenesis System, Promega, Madison, WI), and cloned into the BamHI/ Hind III sites of pSK-GHRH. The GHRH cDNA is followed by the 3' untranslated region of human growth hormone. Endotoxin-free plasmid (Qiagen Inc., Chatsworth, CA, USA) preparation of pSPc5-12-HV-GHRH, pSPc5-12-wt-GHRH and pSPc5-12_gal were diluted in PBS pH=7.4 to 1mg/ml. The animals were assigned equally to one of treatments. The pigs were anesthetized with isoflurane (concentration of 2-6 % for induction and 1-3 % for maintenance). By surgical procedure, we implanted jugular catheters, to drawn blood from these animals at day 3, 7, 14, 21, 28, 45 and 65 post-injection. While anesthetized, 10mg of plasmid was injected directly into the semitendinosus muscle of pigs. Two minutes after injection, the injected muscle was placed in between a set of calipers and electroporated.. At 65 days post-injection, animals were killed and internal organs and injected muscle collected, weighted, frozen in liquid nitrogen, and stored at -80°C.

Porcine GHRH was measured by a heterologous human assay system (Peninsula Laboratories, Belmont, CA). Sensitivity of the assay is 1 pg/tube. Porcine GH in plasma was measured a specific double antibody procedure RIA (The Pennsylvania State University). The sensitivity of the assay is 4ng/tube. Porcine IGF-1 was measured by heterologous human assay (Diagnostic System Lab., Webster, TX). Body composition measurements were performed either *in vivo*, at day 30 and 65 post-injection (densitometry, K40) or post-mortem (organ, carcass, body fat, direct dissection followed by neutron activation chamber). Data are analyzed using Microsoft Excel statistics analysis package. Values shown in the figures are the mean ± s.e.m. Specific p values will be obtained by comparison using Students t test. A $p < 0.05$ was set as the level of statistical significance.

Regulatory regions of most promoters and enhancers often consist of multiple different binding sites for transcription factors. The characteristics of regulatory regions are determined by the composistion and arrangement of the binding sites. Expression vectors have been frequently modified by combining naturally existing promoters and enhancers, and generally these modifications had little or no effect when compared with the transcriptional activity of the native promoters. In addition, available naturally-occuring regulatory regions are not aways capable of regulating transcription in a desired manner.

1) We showed that well understood transcription factors binding elements were incorporated into synthetic promoters. Muscle-specific control elements SRE, MEF-1, MEF-2, TEF-1 and SP-1 linkers were synthesized and randomly assembled. Fragments containing 8-20 control elements represent synthetic promoter/enhancers (SP).

2) We observed that SPc5-12 had a 6 fold increased activity over one of the strongest known promoters, such as CMV, and at least 10 fold greater activity over the skeletal a-actin promoter. Analysis of direct intramuscular injection of DNA plasmids in normal muscle after 2-4 weeks revealed a 4 fold increased activity of SPc5-12 over SK448 promoter and a 10-20 fold increase over the CMV promoter Muscle specificity was confirmed in non-muscle CV1, Hela, 10T1/2 and 293 cell lines and in transgenic animals. Thus, organ-specific promoter/enhancer fragments with an increased activity when compared with the natural promoters can be obtained using this novel strategy that can substantially improve efficiency of transgene expression necessary for gene therapy applications.

3) **GHRH super-active analogs increase GH secretagogue activity and stability.** GHRH has a relatively short half-life of about 12 minutes in the circulatory systems of both humans and pigs. We reasoned that by employing GHRH analogs that prolong its biological half-life and or improve its GH secretagogue activity, we might be able to achieve enhanced GH secretion. Therefore, GHRH mutants were generated by site directed mutagenesis. Substituting Gly15 for Ala15 was used to increase α-helical conformation and amphiphilic structure to decrease cleavage by trypsin-like enzymes. Also, GHRH analogs with Ala15 substitutions display a 4-5 fold greater affinity to the GHRH receptor. To reduce loss of biological activity due to oxidation of the Met, with slightly more stable forms using molecules with a free COOH-terminus, we substituted Met27 and Ser28 for Leu27, Asn28. thus, forming a triple amino acid substitution mutant denoted as GHRH-15/27/28. Dipeptidyl peptidase IV is the prime serum GHRH degradative enzyme. Poorer dipeptidase substrates were created by taking GHRH15/27/28 and then by converting Ala2 for Ile2 (GHRH-TI) or for Val2 (GHRH-TV), or by converting Tyr1 and Ala2 for His1 and Val2 (GHRH-HV; H1V2A15L27N28)).

To test the biological potency of the mutated porcine GHRH cDNA sequences, we engineered plamid vectors that are capable of directing the highest level of skeletal muscle-specific gene expression by a newly described synthetic muscle promoter, SPc5-12 (Li et. Al., 1999). A 228-bp fragment of pGHRH, which encodes the 31 amino acid signal peptide and the entire mature peptide porcine GHRH (Tyr1-Gly40) and or the GHRH mutants, followed by the 3'

untranslated region of hGH cDNA were incorporated into myogenic GHRH expression vectors. Skeletal myoblasts were transfected with each construct and GHRH moieties purified from conditioned culture media cells were assayed for growth hormone secretion in pig anterior pituitary cell cultures. As shown in Figure 1, media collected after 24 hours and quantitated by porcine specific GH-radioimmunoassays showed that modest gains in GH secretion amounting to about 20% to 50% for the modified GHRH species (GH15/27/28; GHRH-TI; GHRH-TV) over wildtype pGHRH. Only one of the four mutants, GHRH-HV, had a substantial increase in GH secretogue activity in which pGH levels rose from baseline values of 200ng/ml up to 1600ng/ml.

Stability of wild type GHRH and the analog GHRH-HV was then tested in porcine plasma, by incubation of GHRH peptides, followed by solid phase extraction, and HPLC, analysis. As shown in Figure 1C, 95% of the wildtype GHRH (1-44)NH_2 was degraded within 60 minutes of incubation in plasma. In contrast, incubation of GHRH-HV in pig plasma showed that at least 75% of the polypeptides was protected against enzymatic cleavage, during 4 to 6 hours of incubation. Thus, under identical conditions, a major portion of GHRH-HV remained intact, while the wild-type GHRH is completely degraded; indicating a considerable increase in stability for GHRH-HV to serum proteases.

4) **Muscle injection of pSP-HV-GHRH increase porcine GHRH; GH and IGF-I serum levels over two months**. We asked if the optimized protease resistant, pSP-HV-GHRH, vector will facilitate long term expression of GHRH and stimulate GH and IGF-I secreted levels. Schematic maps of pSP-HV-GHRH, as well as the wild-type construct, pSP-wt-GHRH, as a wildtype control, and an synthetic myogenic promoter *E.coli.* _-galacosidase expression vector, pSPβgal, as the placebo control,. Three-week-old castrated male-pigs were anesthetized and a jugular vein catheter was inserted, to allow us to collect blood samples with no discomfort for the animals. Plasmid expression vector DNA (10 mg of DNA of pGHRH-HV; pSP-GHRH; pSPβgal was injected directly into semitendinosus muscle, which was placed in between two metal calipers and electroporated, using optimized conditions of 200V/cm with 4 pulses of 60 milliseconds .

The in vivo expression activity of these myogenic GHRH expression vectors was evaluated by measuring their relative GHRH serum levels. In pigs injected with pSP-GHRH-HV, GHRH levels was increased at 7 days post-injection and were 150% above the control levels at 14 days (652.4±77pg/ml versus 419.6±13pg/ml). pSP-GHRH-HV expression activity reached a plateau by 60 days that was about 2 to 3 fold greater levels than the placebo injected control values. The absolute quantity of serum GHRH, corrected for increased body weight between day 0 and day 60 (blood volume accounts for 8% of total body weight), secreted by the pSP-GHRH-HV injected pigs was 3 times greater than the placebo injected control values (1426.49± 10.47ng versus 266.84±25.45ng). The wild-type pSP-GHRH injected animals showed only a modest increase in their GHRH levels starting with 45 days post-injection, but a 2-fold increase by 60 days post-injection (779.36ng), at levels sufficient to elicit a biological effect.

Young animals have very high levels of GH levels that gradually decrease with age. Blood samples, taken every 15 minutes over a 24-hour period after the 7 and 14 days following the initial injections, were assayed for pGH levels (data not shown) which were extraploated for the total change in pGH content. The pGHRH-HV injected pigs (figure 2B) showed an increase in their GH content evident at day 7 post-injection (delta variation HV = +1.52, wt = -0.73 versus control = -3.2ng/ml) and 14 days post-injection (delta variation HV = +1.09, wt = -4.42 versus control = -6.88ng/ml).

Another indication of increased systemic levels of GH would be elevated levels of IGF-I. We observed that serum porcine IGF-1 levels started to rise in pSP-GHRH-HV injected pigs at about 3 days post-injection (figure 3B). At 21 days, these animals averaged about a 3-fold increase in serum IGF-1 levels, which was maintained over 60 days ($p < 0.03$). In comparison, pigs injected with the wild-type pSP-GHRH expression vector had only a 40% increase in their circulating IGF-1 levels ($p = 0.39$).

Porcine GH secreted into the systemic circulation after intramuscular injection of myogenic pSP-GHRH expression vectors augments growth over 65 days in castrated young male pigs. Wild-type pSP-GHRH injected animals were on average 21.5% heavier than the placebo controls (37.125kg vs. 29.375kg), while the pSP-GHRH-HV injected pigs were 37.8% heavier (41.775kg; $p = 0.014$). Feed efficiency is also improved by 20% in pigs injected with GHRH constructs, when compared with controls (0.267 kg of food/day for each kg weight gain in pSP-HV-GHRH, and 0.274 kg in pSP-wt-GHRH, versus 0.334 kg in pSP__gal injected pigs. Body composition studies by densitometry, K40 potassium chamber and neutron activation chamber showed a proportional increase of all body components in GHRH injected animals, with no signs of organomegaly, relative proportion of body fat and associated pathology. A photograph of a placebo injected control pig and a pSP-GHRH-HV injected pig after 45 days is shown below.

The metabolic profile of pSP-HV-GHRH injected pigs connotes a significant decrease in serum urea level (9±0.9mg/ml in controls, 8.3±1mg/ml and 6.875±0.5mg/ml in injected pigs, pSP-GHRH and pSP-GHRH-HV, respectively ($p=0.006$), indicating decreased amino acid catabolism. Serum glucose level was similar in between the controls and the plasmid GHRH injected pigs (99.2±4.8mg/ml in control pigs, 104.8±6.9mg/ml in pSP-GHRH-HV injected pigs and 97.5±8mg/ml in wildtype pSP-GHRH injected animals ($p= 0.263$). No other metabolic changes were found.

We have recently shown that defective SRF protein results in less transactivation of the skeletal α-actin gene. Dr. Frank Booth and colleagues have previously shown decreases in the concentration of the skeletal "-actin mRNA in unloaded soleus and gastrocnemius muscles of rats. They have also shown increases in SRF protein mass per unit of muscle protein in hypertrophying skeletal muscle. We hypothesized that SRF protein would decrease in unloaded muscles. We found that SRF protein mass per unit of total protein decreased in both soleus and plantaris muscles of 7-day hindlimb unloaded rats. In addition, Booth Frank previously showed

that myogenin mRNA increased during muscle hypertrophy and hypothesized that myogenin mRNA would increase in soleus muscle regrowth from spaceflight or from hindlimb unloading. On the first day of recovery from either spaceflight or from hindlimb unloading, myogenin mRNA was increased in the soleus muscle (Funded exclusively by NASA). (Funded exclusively by NASA).

Dr, Frank Booth's group have previously shown that focal adhesion kinase and paxillin protein masses per unit of total protein has a transient one-week increase in hypertrophying skeletal muscle. From this we hypothesized that focal adhesion kinase and paxillin protein masses per unit of total protein would decrease in atrophying skeletal muscle. While we noted this effect in 7-day unloaded fast-twitch plantaris and gastrocnemius muscles, we observed an increase in focal adhesion kinase and paxillin protein masses pre unit of total protein in the 7-day unloaded soleus muscle (Funded exclusively by NASA)..

Knockout of myostatin gene expression produces muscle hypertrophy in transgenic mice and existing mutations in cows. Dr. Frank Booth hypothesized that increased myostatin mRNA would occur in unloaded soleus muscles. Myostatin mRNA increases transiently in the gastrocnemius (fast-twitch) muscle during hindlimb unloading of mice. Intriguingly, we were unable to detect myostatin mRNA in either the control or unloaded soleus muscle. Remarkably, myostatin mRNA was highest in type IIb muscle fibers of mice and correlated with type IIb fiber percentage. Myostatin mRNA is highest in the largest diameter muscle fibers of adult mice. While myostatin mRNA may have a role in muscle development, its role in skeletal muscle of adult mice is less certain (mainly funded by NIH).

II. IMPLICATIONS OF PROJECT FINDINGS FOE FUTURE RESEARCH

We believe that this novel system of designing synthetic promoter/enhancer using rather individual regulatory sequence than entire promoters represents a significant improvement over previously generated plasmid DNA expression vectors. Optimization of plasmid DNA vectors for gene therapy. should increase their utility for systemic (hormones or vaccines) or local (neurotrofic factors for the treatment of neurodegenerative diseases or injured muscle) delivery of therapeutic proteins as hormones or coagulation factors, or to locally deliver proteins, as growth factors for treatment of crushed and injured muscle or neurodegenerative diseases

Ectopic expression of a novel serum protease resistant porcine growth hormone releasing hormone, directed by an injectable muscle synthetic promoter plasmid vector, pSP-GHRH-HV, elicits profound growth in pigs over 2 months. Employing electrogene therapy, which does not require viral genes or particles, allows genes to be transferred and expressed in desired organs or tissues, and it may lead to the development of a new type of highly effective gene therapy. Although, the exact mechanism for enhanced DNA uptake in muscle is not yet known, it is thought that change in polarity increases the plasmid DNA uptake probably by opening of membrane pores and that repetitive depolarizations uniformly spreads DNA throughout the muscle by forming transient protein DNA complexes. The electroporation system has been previously used in rodents and small animals and does cause any serious discomfort. In

combination with improved muscle expression vectors electrogene therapy with needle electrodes increases vector activity over 5,000-10,0000 fold and allowed for prolonged GHRH-HV expression over 60 days in pigs

Enhancing biological potency reduces the theoretical quantity of GHRH plasmid needed to achieve physiological levels of GH that are necessary for animal gene therapy. The treated pigs did not experience any side effects from the therapy, have normal biochemical profiles, with no associated pathology and no organomegaly. The profound increases in IGF-I levels and resulting growth enhancement in growth over two months indicates that ectopic expression of myogenic GHRH-HV vectors via electrogene treatment may replace classical GH protein regimens and has the potential to stimulate GH axis in a more natural fashion. We predict that the GHRH-HV species which display a high degree of stability and GH secretory activity in pigs, might also be employed in human clinical medicine, since the serum proteases that turn-over GHRH are similar in most mammals.

Our study will provide a framework against which other types of therapies can be compared, both in clinical studies on GH deficient children or in elderly, where our therapy could be combined with an adequate diet for optimized results in growth, muscle strength and/or bone mineralization. As a key hormone replacement therapy, induced secretion of GH by GH secretogoues, has been studied for its potential on improving nutrition, skeletal growth and maintenance of muscle homeostatsis.

We will continue upon our proposed studies to determine if the upregulation of the GHRH/GH/IGF-I axis will act in synergy with resistive exercise regimens to block muscle atrophy under microgravity. Enhancement in muscle mass could occur by GHRH secretagogue activity increasing the systemic levels of growth hormone, which then elicits increased level of systemic or local IGF-I production in muscle. It is very important to note that space flight conditions induce stress to some degree by heavy work loads, lack of sleep, impaired circadian daylight cycles and nutrition. Hormones secreted in greater amounts as part of the stress response include ACTH and cortisol which increased greater than 100% during Skylab missions Cortisol is one of the primary muscle catabolic factors that rapidly elicits muscle protein break down and impairs GH secretion and reduces IGF-I levels, increases bone loss and damages the immune system. Also, when adult rats were treated with dexamethasone, IGF-I was still able to stimulate protein synthesis in the epitrochlearis muscle, but only by 41.9% of the increase found in a pair-fed control group (Dardevet et al. *J. Endocr.*. 156:83, 1998). As hindlimb unloaded mice should have increases in plasma glucocorticoids, it is possible that this would suppress the action of IGF-I. Furthermore, Dr. Fred Goldberg showed three decades ago that inactive skeletal muscle becomes more sensitive to glucocorticoids (*J. Physiol.* 200:667, 1969).). Thus, we will also plan to test the myogenic injectable GHRH vector system in rats treated with glucocorticoids as a therapy to block steroid induced muscle catabolism.

Clearly, development of the injectable GHRH gene therapy has great synergy with the goals set forth for human performance and bone teams. It is up to the NSBRI and the NASA

A

Element	Pools of regulatory elements					
	1	2	3	4	5	6
SRE	1	4	1	1	1	4
MEF-2	1	1	4	1	1	1
MEF-1	1	1	1	4	1	4
TEF-1	1	1	1	1	4	1

B

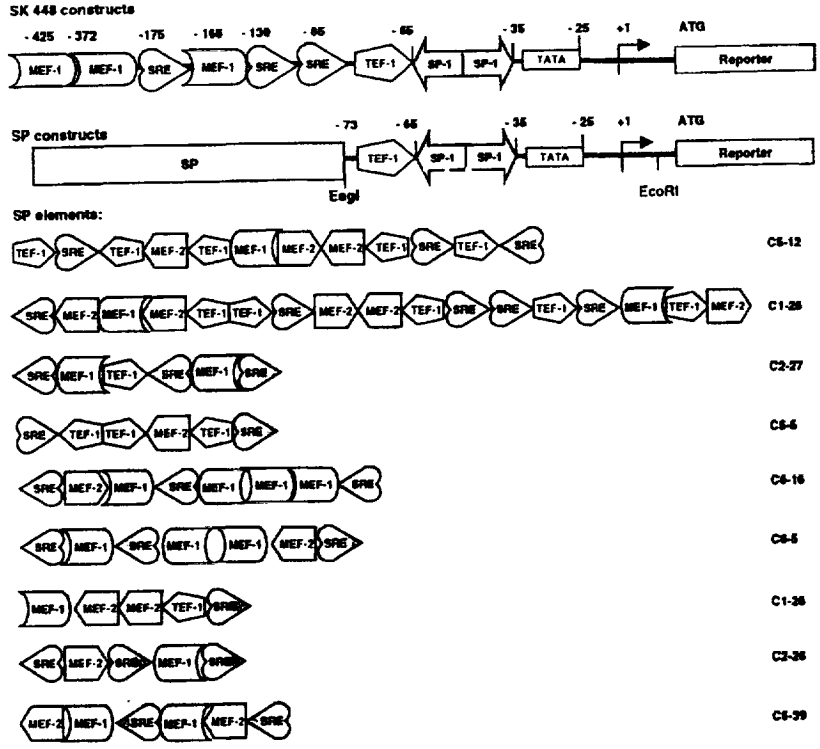


Figure 1. Synthetic promoter design and elements. A. Proportion of regulatory elements in different combinations of synthetic promoters; each combination contains at least one of each muscle specific regulatory elements. **B.** Synthetic promoter elements in the constructs with the highest *in vitro* reporter gene activity compared with skeletal *a*-actin 448 promoter; most of these SP were found in pools of elements 1, 5 and 6 (e.g. combination 1: the proportion between SRE: MEF-2: MEF-1: TEF-1: SP1 was 1:1:1:1:4; in combination 5-1:1:1:4:6 and in combination 6- 4:1:1:4:1:6).

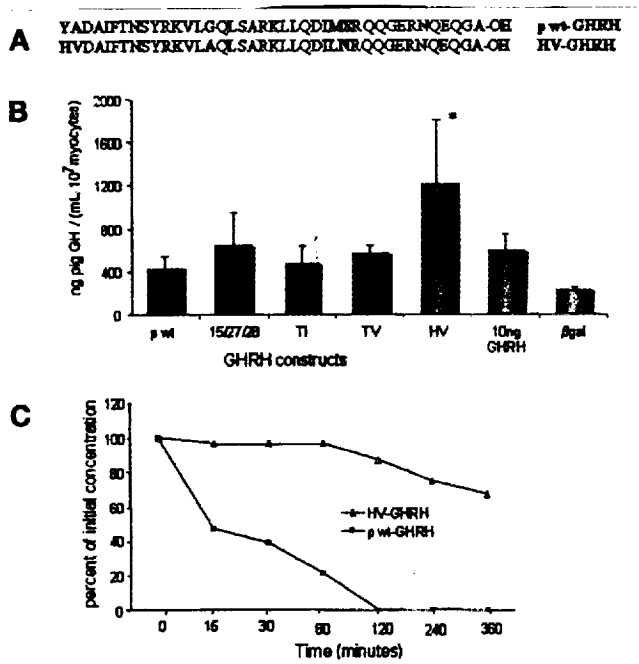


Figure 2. GHRH super-active analogs increase GH secretagogue activity and stability. A. All myogenic expression vectors contain the SPc5-12 synthetic promoter and the 3'UTR of hGH cDNA. As a model of mutated protein, HV-GHRH construct was used, and compared with the porcine wild-type as a positive control, and with _-galactosidase construct, as a negative control. **B.** Comparison of the porcine wild-type GHRH (Tyr1-40) amino acid sequence with the analog, GHRH-HV **C.** Pig GH release in porcine primary pituitary culture is simulated by GHRH species isolated from conditioned media of skeletal muscle cells transfected with myogenic expression vectors driving porcine GHRH analogs. Porcine wild-type GHRH (Tyr1-40) construct is represented by pwt., Amino acid substitutions of Gly15 to Ala, Met27 to Leu and Ser28 to Asn is represented by GHRH-15/27/28. Substitutions as in 15/27/28, plus the conversion of Ala2 to Ile2 is represented by GHRH-TI. Substitutions as in 15/27/28, plus the conversion of Ala2 to Val2 is represented by GHRH-TV. Substitutions as in 15/27/28 plus conversion of Tyr1 with His, and Ala2 with Val is represented by GHRH-HV, The construct coding for E.coli beta-galactosidase,beta gal, is used as a negative control. Cells were stimulated with 10 ng of recombinant hGHRH (Tyr1-44).

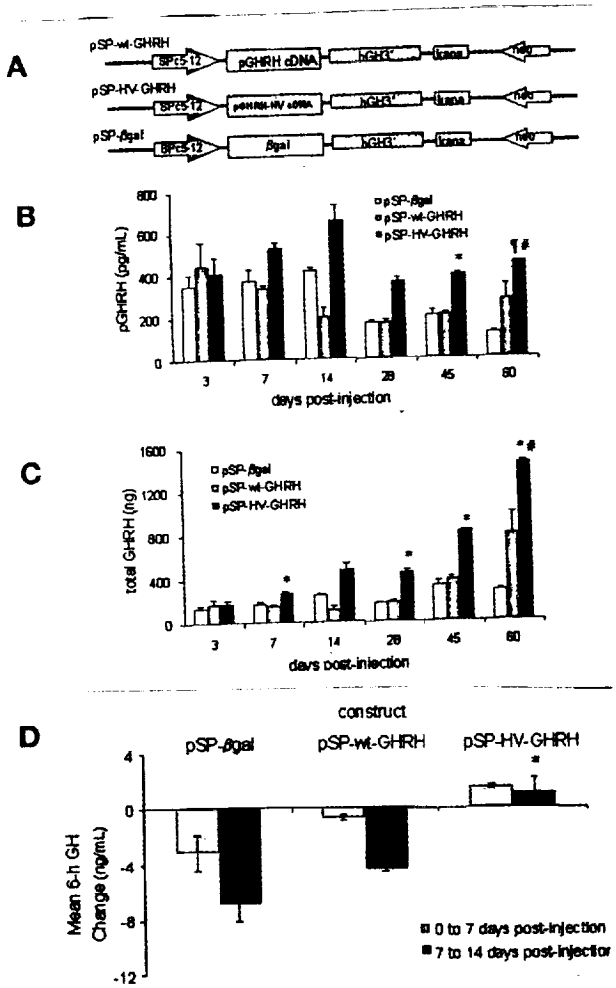


Figure 3. Single injections of super analog GHRH myogenic expression vector increases porcine GHRH; GH and IGF-I serum levels over two months. **A.** Relative levels of serum GHRH levels in pSP-GHRH injected pigs versus placebo injected control pigs. **B.** Absolute levels of serum GHRH in pSP-GHRH injected pigs versus control pigs corrected for weight/blood volume increase. **C.** pSP-HV-GHRH injected pigs show a positive variation of GH levels. **D.** Plasma IGF-1 level after direct intramuscular injection of pSP-GHRH constructs.

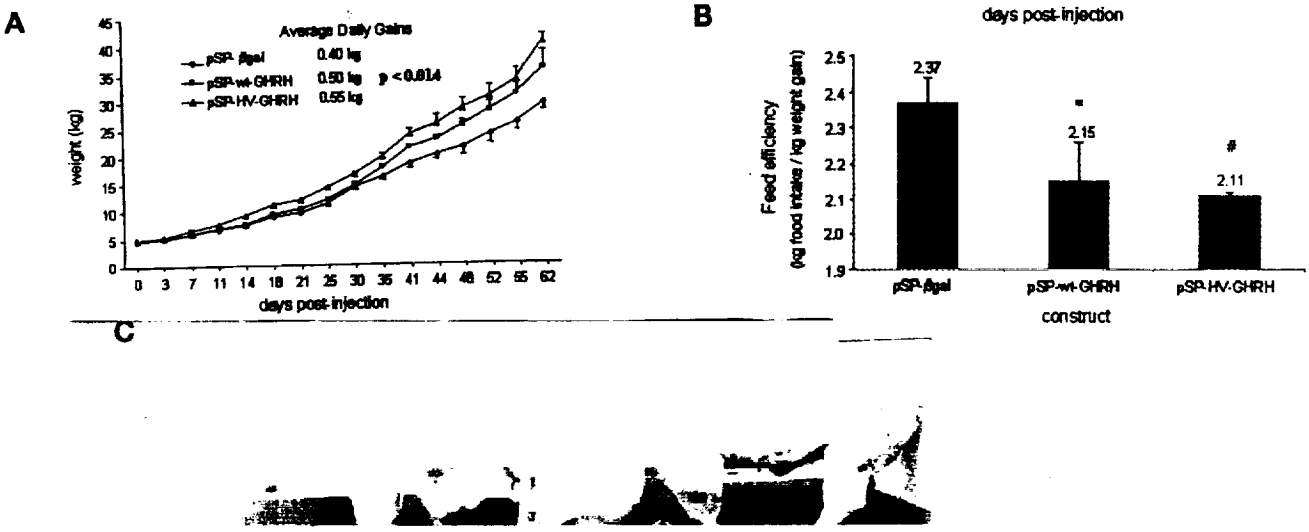


Figure 4 Myogenic GHRH expression vectors enhance pig growth. **A.** Average weight increase in injected pigs over 2 month after pSP-GHRH and pSP-GHRH-HV. **B.** Improved feed efficiency in the pSP-GHRH injected pigs versus controls. **C.** Comparison of a pSP-HV-GHRH injected pig and a placebo injected control pig, 45 days post-injection.

B .List of publications supported through NSBRI funding and appropriately acknowledged.

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The Activation Of Protein Breakdown In Muscle Upon Unloading And Possible Countermeasures

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I. EXECUTIVE SUMMARY

RESEARCH PROJECTS (1997-2000)

1) Critical Role of Certain Ubiquitination Enzymes

Various prior observations had suggested that the increased protein breakdown responsible for muscle atrophy in many diseases (e.g. cancer, sepsis, diabetes, or hyperthyroidism) is primarily due to an activation of the Ub-proteasome pathway. During the past three years using homogenates of normal and atrophying muscles, we showed that overall rates of Ub-conjugation increase in atrophying muscles, and these hormone and cytokine-dependent responses are due largely to activation of the N-end rule pathway for Ub-conjugation. Specifically we discovered that Ub-conjugation to endogenous proteins and to a model substrate of the N-end rule pathway (lysozyme) were significantly increased. We found that mRNA for the critical ubiquitination enzymes in the N-end rule pathway, $E2_{14k}$ and E3α, are increased about 2-fold in the atrophying muscles. Thus, the activation of Ub-conjugation and proteolysis by the "N-end" pathway appears to be a very general feature of atrophying muscles, independent of the cause.

In normal muscle extracts, we found that the N-end rule pathway for ubiquitin conjugation also appears to be responsible for the degradation of most soluble proteins. In contrast to muscle, in extracts of HeLa cells, this system is also present but makes only a minor contribution to overall protein ubiquitination. These findings were quite unexpected, because the ubiquitinating enzymes comprising the N-end rule pathway, and in particular E3α, are believed to recognize abnormal proteins with unusual amino-terminal residues. An important role for $E2_{14k}$ and E3α in muscle (or in any other cell) was unanticipated since the synthesis of all proteins begins with a methionine in the N-terminus, and over 80% have their N-termini acetylated, which prevents recognition by the E3α.

2) Ubiquitination Conjugation After Hind-Limb Suspension.

A similar increase in ubiquitin conjugation was found in extracts of muscle following hind-limb suspension (performed by our collaborator, Dr. Ken Baldwin). These findings suggest that the disuse atrophy in astronauts involves a similar activation of the Ub-proteasome pathway, as we have found in many other types of muscle wasting (although these changes upon hind-limb suspension were less pronounced and therefore harder to study routinely than in some other atrophying models).

3) Possible Use of Protease inhibitors

One other goal of our work during the initial grant period was to evaluate the possible utility of the newly discovered proteasome inhibitors as possible countermeasures to retard the excessive protein breakdown in atrophying muscles. It is noteworthy that these inhibitors, especially the peptide aldehydes, had much larger effects in reducing proteolysis in atrophying muscles than in control muscles. Thus, as suggested previously, the enhanced proteolysis in many catabolic states is due to a proteasome-dependent pathway. Because inhibition of proteasome function in principle could be a useful approach to reduce muscle wasting, we carried out systematic studies to define the effects of peptide aldehydes and the more selective proteasome inhibitor, lactocystin-β-lactone on intracellular proteolysis. These findings provided definitive support for our prior conclusions that the proteasome is the site for degradation of long-lived as well as

short-lived proteins. On the other hand, we and a number of other investigators have found that prolonged exposure of most cells to these various inhibitors (i.e. for 24-32 hours) at concentrations that cause a large reduction in protein breakdown causes cell death by apoptosis. So, although partial inhibition of proteasome function is well tolerated, complete inhibition is dangerous, therefore these types of inhibitors are inappropriate for use by astronauts as countermeasures and can only be used against life-threatening illnesses in a hospital setting. In fact, such clinical trials are in progress now in cancer patients. Consequently, future studies will focus on development of inhibitors of ubiquitination enzymes that are specifically important in atrophy (whose inhibition should be non-toxic and affect primarily atrophying muscles).

4) Patterns of Gene Expression in Atrophying Muscles

It seems likely that (1) muscle atrophy involves a suppression of the same program of gene expression that is activated during work-induced hypertrophy or by IGF in normal growth and (2) that there also exist a number of genes induced during muscle atrophy that are of major importance in the loss of muscle protein and contractile function. Our studies thus far have uncovered several genes (e.g. Ub, proteasome subunits, etc.), whose expression increases approximately 2-3 fold in all types of muscle atrophy studied thus far. The increases in these mRNAs are noteworthy because atrophying muscles generally show a decrease in total mRNA and ribosomal RNA. We propose to call such genes, atrophy genes, since these gene products which we call "atrophins" are likely to play a key role in the process of muscle wasting.

Because of the potential value of information on the nature of these atrophy genes, during the past year, under the NSRBLI grant we have initiated a gene microarray analysis to obtain a comprehensive picture of the transcriptional changes occurring during muscle atrophy. This approach allows comparison on a single chip of mRNAs from experimental and control tissues from mouse or human cDNA libraries containing 5-10,000 different cDNAs. In order to validate this approach, in our initial experiments we chose to study the pattern of changes in muscle mRNAs induced by fasting, primarily because of the simplicity of this model and the wealth of prior information on this type of muscle wasting, especially the changes in proteolysis and energy metabolism. Our initial observations have proven very informative and promising. We have found over 100 genes whose levels change by about 2-fold or more in fasting. They fall into several categories including mRNAs encoding a) multiple subunits of the 20S proteasome and its 19S regulatory complex, which are coordinately up-regulated (as expected). Most interestingly, there are 7 genes (ORFs) whose expression increases most markedly (4-9 fold), and surprisingly, the functions of all of them are unknown. We are beginning to clone the most highly induced species in order to analyze their expression, to see if they are induced upon unloading, to prepare antibodies against the encoded proteins, and to explore their functions. The protein encoded by this most highly regulated mRNA, which we term atrophin-1, resembles that a subunit of a new type of E3 (a ubiquitination protein ligase) belonging to the F-Box. These exciting observations suggest that this protein is part of a new ubiquitination enzyme involved in the acceleration of protein breakdown during muscle wasting.

II. PROJECT RESEARCH ACTIVITY AND IMPLICATIONS OF FINDINGS FOR FUTURE RESEARCH

FINDINGS MADE WITH SUPPORT OF THE NSBRI (1997-2000)

During the past 3 years with support from the NSBRI, we have achieved major progress in all the studies proposed in our 1997 application. In addition, certain very promising new studies were initiated that were aimed toward development of novel countermeasures and to test the relevance of our findings on rats to combat muscle wasting in humans. These various developments are summarized below.

A) The N-end rule pathway for ubiquitin conjugation catalyzes a major fraction of the protein degradation in skeletal muscles. One property of a protein that leads to its rapid degradation by the Ub-proteasome pathway is the presence of a basic, acidic, or bulky hydrophobic residue at its amino terminus (the N-end rule pathway for ubiquitin conjugation). However, in normal cells, the origin and abundance of substrates for this ubiquitination system, which involves E2$_{14k}$ and E3α, have remained unclear. Surprisingly, in soluble extracts of rabbit muscle, we found that competitive inhibitors of E3α (dipeptides and amino acid esters with basic or hydrophobic N-termini) blocked up to 60% of the ATP-dependent degradation of endogenous proteins and inhibited similarly their conjugation to ^{125}I-Ub. These agents (but not isomeric dipeptides or other amino acid esters) selectively inhibit E3α and blocked the degradation of model substrates of the N-end rule pathway for ubiquitin conjugation but did not affect degradation of proteins whose ubiquitination involved other E3s. Also, a similar general inhibition of ^{125}I-Ub conjugation to endogenous proteins was observed with a dominant negative inhibitor of E2$_{14k}$. Thus, the N-end rule pathway for ubiquitin conjugation appears to be responsible for the degradation of most soluble proteins in muscle extracts. In contrast to muscle, in extracts of HeLa cells, this system is also present but makes only a minor contribution to overall protein ubiquitination.

B) In muscle atrophy induced by various stimuli, rates of Ub-conjugation increase largely through activation of the N-end rule pathway. Various observations had suggested that the increased protein breakdown in muscle in many diseases (e.g. cancer, sepsis, or hyperthyroidism) is primarily due to an activation of the Ub-proteasome pathway. In extracts of atrophying muscles from tumor-bearing and septic rats, rates of ^{125}I-Ub-conjugation to endogenous proteins were faster than in control extracts. On the other hand, in extracts of muscles from hypothyroid rats, where overall proteolysis is reduced below normal, ^{125}I-Ub-conjugation to soluble proteins decreased by 50%, but treatment with triiodothyronine (T_3) restored ubiquitination toward control levels. Surprisingly, the N-end rule ubiquitination pathway seems responsible for most of these changes in Ub-conjugation. Specific dipeptide or amino acid ester inhibitors of the Ub-protein ligase, E3α, suppressed most of the increased Ub-conjugation in the extracts from the atrophying muscles and septic rats. These inhibitors also reduced ubiquitination in normal extracts to levels in hypothyroid extracts, which showed little E3α-dependent ubiquitination. Thus, the inhibitors of E3α eliminated most of the differences in ubiquitination under these different conditions. Moreover, ^{125}I-lysozyme, a typical N-end rule

substrate, was ubiquitinated more rapidly in extracts from tumor-bearing and septic rats and more slowly in those from hypothyroid rats than in controls. Thus, overall rates of Ub-conjugation increase in atrophying muscles, and these hormone and cytokine-dependent responses are due largely to activation of the N-end rule pathway for Ub-conjugation.

More recently, in collaborative studies with Lazarus et al (38), we have extended these observations to a new model of cancer cachexia in the mouse, which unlike the tumor-bearing or septic rats studied above, is not triggered by the cytokine, TNF. After tumor implantation, body and muscle weights decreased by 20-25%, despite normal food intake, and there was a loss of myofibrillar proteins. As observed above, Ub-conjugation to endogenous proteins and to the model substrate of the N-end rule pathway lysozyme were significantly increased. Thus, the activation of Ub-conjugation by this pathway appears to be a very general feature of atrophying muscles, independent of the cause.

Implications. These findings were quite unexpected, because the ubiquitinating enzymes comprising the N-end rule pathway, and in particular E3α, are believed to recognize abnormal proteins with unusual amino-terminal residues (i.e. basic and large hydrophobic residues). An important role for $E2_{14k}$ and E3α in muscle (or in any other cell) was unanticipated since the synthesis of all proteins begins with a methionine in the N-terminus, and over 80% have their N-termini acetylated, which prevents recognition by the E3α. Exactly why $E2_{14k}$ and E3α are so important in muscle and how and why these particular ubiquitinating enzymes are activated in atrophying tissues are important questions that we hope to resolve. It remains possible that $E2_{14k}$ and E3α may function in muscle through an unappreciated capacity to recognize muscle proteins by some internal site in their sequences. Alternatively, the finding that the N-end rule pathway is very important in the accelerated proteolysis in atrophying muscle raises the novel possibility that the first step in degradation of muscle proteins might be a modification of cell proteins, either by an endoproteolytic cleavage or an N-terminal deacetylation, which exposes free amino groups leading to their recognition and ubiquitination by E3α (Figure 4). Since these observations seem key to understanding how proteolysis may be activated in the atrophying tissues, we have begun developing assays to characterize the N-termini of ubiquitinated proteins in muscle to see if they indeed conform to the preferences of the N-end rule and to determine whether the nature of the N-termini on muscle proteins changes during atrophy. In future experiments, we hope in coming years to test whether there is an initial endoproteolytic cleavage or a deacetylation reaction that triggers protein recognition by the N-end rule pathway in atrophying muscle.

C) Ubiquitin conjugation is increased in the hind-limb suspension model of weightlessness. Extracts of muscles prepared from rats after hind-limb suspension (provided by our collaborator, Dr. Kenneth Baldwin) were used to test whether these same mechanisms also function in disuse atrophy. When these soleus muscles were incubated *in vitro*, they showed increased rates of protein degradation by the nonlysosomal ATP-dependent pathway and increased levels of mRNA encoding polyubiquitin. These results are typical of earlier findings in other types of atrophy and support our earlier suggestion that atrophying muscles show a common series of adaptations that activate this degradative pathway (whatever may be the specific cause of the atrophy). One week after hind limb suspension, there was a 2-fold increase in the overall rate of ^{125}I-Ub conjugation to endogenous proteins in extracts from the soleus, the antigravity muscle most sensitive to unloading-induced atrophy (Figure 1). These findings suggest that the disuse

atrophy in astronauts involves a similar activation of the Ub-proteasome pathway, as we have found in many other types of muscle wasting (although these changes upon hind-limb suspension were less pronounced and therefore harder to study routinely than in some other atrophying models). Therefore, most of our further experiments (e.g. on the time course of the response to unloading) were postponed until we developed more sensitive tools to monitor changes in activity and expression of various components of the N-end rule. Such tools are now available and will used in coming years.

D) Changes in Individual enzymes of the N-end rule pathway during atrophy. Since the N-end rule pathway plays a major role in ubiquitin conjugation in atrophying muscle, we tested whether its activation is due to induction of $E2_{14k}$ and E3α. As a convenient experimental system for muscle wasting, we studied muscles from rats made insulin-deficient by the administration of streptozotocin. The resulting deficiency in insulin and secondarily in IGF-1 in such animals (as in fasting animals) causes a rapid muscle wasting due largely to enhanced protein breakdown that is dependent on the rise in circulating glucocorticoids and is blocked by treatment with insulin. We also believe that this experimental model may be relevant to space personnel, since low insulin levels have been noted in some astronauts and reduced contractile load and glucocorticoids are known to make skeletal muscle resistant to insulin. We found that mRNA for both $E2_{14k}$ and E3α are increased about 2-fold in the atrophying muscles (Figure 2), and similar changes have also been seen by others in muscles of hind-limb-suspended rats and in rats with sepsis. Surprisingly, however, in these same muscles increases in the levels of $E2_{14k}$ and E3α protein or in their activity were not detected by Western blot with antibodies we generated. Possibly the increases in $E2_{14k}$ and E3α protein are below the level of detection in these assays, but it is also possible that small changes in each component together can lead to increased ubiquitin conjugation. Alternatively, other, as yet undefined, factors may be acting in conjunction with these components to lead to enhanced Ub-conjugation by the N-end rule pathway.

E) Studies of the ability of proteasome inhibitors to retard protein breakdown in normal and atrophying muscles. One goal of our work during the initial grant period was to evaluate the possible utility of the newly discovered proteasome inhibitors, especially the peptide aldehydes, as possible countermeasures to retard the excessive protein breakdown in atrophying muscles. In addition, such studies represented a definitive test of the importance of the Ub-proteasome pathway in the excessive proteolysis we had previously found in various catabolic states. A number of observations had suggested that the atrophy of disused muscles and in various systemic diseases is due primarily to activation of the ubiquitin-proteasome pathway. To test this idea, we investigated whether peptide aldehyde inhibitors of the proteasome (LLN or the more potent MG132) suppressed proteolysis in incubated rat skeletal muscles. These agents (e.g., MG132 at 10 μM) inhibited nonlysosomal protein breakdown by up to 50% ($P<0.01$), and this effect was rapidly reversed upon removal of the inhibitor.

While retarding proteolysis, the peptide aldehydes did not alter protein synthesis or amino acid pools, but improved overall protein balance in the muscle. As expected, upon treatment with MG132, ubiquitin-conjugated proteins accumulated several fold in the muscle. The inhibition of muscle proteolysis correlated with efficacy against the proteasome, (although these peptide aldehydes could also inhibit calpain-dependent proteolysis induced with Ca^{2+} ionophores).

It is noteworthy that these inhibitors had much larger effects on proteolysis in atrophying

muscles than in controls. In the denervated soleus undergoing atrophy, the increase in ATP-dependent proteolysis was reduced 70% by MG132 ($P<0.001$). Similarly, the rise in muscle proteolysis induced by administering thyroid hormones was reduced 40-70% by the inhibitors. Finally, in septic rats, the increase in muscle proteolysis was completely blocked by MG132. In related experiments in collaboration with W. Mitch, MG 132 was found to selectively inhibit the increased muscle proteolysis in diabetic and acidotic animals. Thus, as suggested previously, the enhanced proteolysis in many catabolic states is due to a proteasome-dependent pathway. In addition, inhibition of proteasome function in principle could be a useful approach to reduce muscle wasting.

F) Novel proteasome inhibitors. Additional studies probed the effects of other selective inhibitors of proteasome function. Because lactacystin β-lactone appeared to be a more specific inhibitor than the peptide aldehydes, and because lactacystin derivatives are in human clinical trials, we carried out systematic studies to define its effects on intracellular proteolysis. These findings provided definitive support for our prior conclusions that the proteasome is the site for degradation of long-lived as well as short-lived proteins. Using lactacystin on incubated muscles, we confirmed our prior suggestion that the Ub-proteasome pathway was responsible for most protein breakdown in muscle. The lactone was also found to reduce the excessive degradation in atrophying muscles (e.g. ones from tumor bearing animals). However, rodent muscles required much higher doses of this inhibitor (>50μM) than many other cell types studied, presumably because of problems with its transport.

G) Other Effects of Proteasome Inhibitors. Because this capacity of the proteasome inhibitors to reduce overall proteolysis in isolated skeletal muscles appeared to have therapeutic potential, additional experiments investigated whether longer term exposures might have deleterious or protective effects. An accumulation of misfolded proteins in cells is known to lead to the expression of heat shock proteins and molecular chaperones in the ER. Therefore, we tested whether these inhibitors, by causing an accumulation of abnormal proteins, might stimulate the induction of these stress proteins. Exposure of cultured cells to various proteasome inhibitors inhibited the degradation of short-lived proteins and increased markedly the levels of mRNAs encoding heat-shock proteins, as shown by Northern blot analysis. However, inhibitors of other proteases had no effect. The relative efficacies of the inhibitors in inducing these mRNAs correlated with their potencies against the proteasome. However, the proteasome inhibitors did not affect total protein synthesis, protein secretion, ER morphology, or the retention of ER-lumenal proteins, even after 18h of treatment. Together, the findings suggest that inhibition of proteasome function induces a cellular stress response without other signs of cell injury.

Since expression of heat-shock proteins can protect cells from thermal and oxidative injury, we tested whether exposure to these inhibitors might also confer thermotolerance. Treatment with MG132 for as little as 2h, markedly increased the survival of cells subjected to otherwise lethal temperatures (up to 46°C). Moreover, in related experiments with other cell types, we found that treatment with proteasome inhibitors also enhances dramatically cell resistance to oxygen free radicals. This unexpected discovery (that short exposure to these agents can protect against acute cell injury), in fact, may account for the ability of the proteasome inhibitors to reduce ischemic injury in experimental animals.

On the other hand, a number of investigators have found that prolonged exposure of most cells to these various inhibitors (i.e. for 24-32 hours) at concentrations that cause a large

reduction in protein breakdown causes cell death by apoptosis. However, when administered carefully in animals and human clinical trials, these agents have been given repeatedly for many weeks at doses that inhibit proteasome function without apparent toxicity. So, partial inhibition of proteasome function is well tolerated.

NEW DEVELOPMENTS

Gene Microarray Analysis of Muscle Atrophy.

Rationale. Our studies thus far have uncovered several genes (e.g. Ub, proteasome subunits, etc.), whose expression increases approximately 2-3 fold in all types of muscle atrophy studied thus far. The increases in these mRNAs are noteworthy because atrophying muscles generally show a decrease in total mRNA and ribosomal RNA. We propose to call such genes, atrophy genes, since they are likely to play a key role in the process of muscle wasting. The expression of these specific genes was originally studied, because their induction might enhance the cell's proteolytic capacity. However, there must also exist a number of other atrophy genes that remain to be discovered and that are of equal or greater importance in the loss of muscle protein and contractile function. Identification of additional atrophy-genes should advance our understanding of key steps in this process and could suggest new targets for pharmacological intervention.

During atrophy, there must also occur a marked decrease in the expression of many genes in the muscle, since overall protein synthesis and synthesis of myofibrillar proteins decrease upon unloading and in most other types of atrophy. For example, upon hind-limb suspension mRNA for Type 1 myosin decreases, and this effect contributes to the shift in fiber type specificity with unloading. One attractive hypothesis is that muscle atrophy involves a suppression of the same program of gene expression that is activated during work-induced hypertrophy or by IGF in normal growth. Recently several proteins (e.g. calcineurin and NFAT) have been shown to play key roles in such growth of cardiac and skeletal muscle. Clearly, it will be of appreciable interest to learn whether the mRNAs for these important regulatory proteins and for proteins involved in muscle energy metabolism and Ca^{2+}-regulation actually decrease selectively in atrophying muscles.

Because of the potential value of such information, during the past year, under the NSRBLI grant we have initiated a gene microarray analysis to obtain a comprehensive picture of the transcriptional changes occurring during muscle atrophy. Traditional approaches to measuring the expression of specific genes (e.g. Northern blotting and RNAse protection assays) are only suitable for monitoring small numbers of genes. This new "gene-chip" technology has enabled us to monitor the differential expression of several thousand genes in the same experiment, and has already been used successfully by a number of labs (e.g. to analyze disease-related genes). While microarrays lack the sensitivity of many assay methods, they allow the investigator to establish which mRNAs are coordinately regulated and to find unknown genes involved in a biological process. Harvard has recently established its own Center for Genomics Research, which both provides access to microarray gene analysis and expertise in its use and data-analysis. Our lab has been one of the first to use this facility; we have employed the microarray system developed by Incyte Inc, which allows comparison on a single chip of mRNAs from experimental and control tissues that bind from mouse or human cDNA libraries containing 5-10,000 different cDNAs.

In order to validate this approach, in our initial experiments we chose to study the pattern of changes in muscle mRNAs induced by fasting, primarily because of the simplicity of this model and the wealth of prior information on this type of muscle wasting, especially the changes in proteolysis and energy metabolism. We used this model to explore reproducibility of data obtained by this approach, to compare data from the gene microarrays with data obtained by Northern Blot analysis, and to compare the utility of mouse and human cDNA libraries (both of which gave useful, complimentary data).

Preliminary results. Our initial observations have proven very informative and promising. They have shown that highly reproducible patterns can be obtained with RNA from ten mouse muscles and that valuable, non-redundant information can be obtained in mouse muscles by use of both human and mouse chips (which encode distinct cDNAs) (Figure 3). Although data generation is relatively easy, the analysis of the results by Dr. Jagoe has required several months of work to organize, identify cDNAs, and analyze probable physiological functions of encoded proteins. We have found over 100 genes whose levels change by about 2-fold in fasting. They fall into several categories including mRNAs encoding a) multiple subunits of the 20S proteasome and its 19S regulatory complex, which are coordinately up-regulated (as expected) (Figure 4), b) many myofibrillar proteins, glycolytic enzymes and mitochondrial components, which are down-regulated (Figures 5 & 6). c) some proteins involved in transcription and translation are increased and some decreased, presumably because of their specific regulatory roles, and e) a few genes of diverse function (e.g. certain cytoskeletal components), whose induction in atrophying muscles is unexpected and unexplained.

Most interestingly, there are 7 genes (ORFs) whose expression increases most markedly (4-9 fold), and surprisingly, the functions of all of them are unknown. Because the identification of these highly regulated components could be especially informative, we have begun to clone the most highly induced species in order to analyze their expression, to see if they are induced upon unloading, to prepare antibodies against the encoded proteins, and to explore their functions. We have initially focused on the EST that we named Atrophy Gene-1 (AG-1), whose mRNA showed a ~9-fold increase upon fasting. Moreover, using this cDNA as a probe in a Northern blot analysis, we detected 3 bands, whose signals also increased up to 4-fold in atrophying muscles from tumor-bearing (cachectic) and diabetic rats. (Experiments with unloaded muscles are in progress.) After conducting sequence analysis using LabOnWeb algorithms, we identified probable 3' and 5 sequence extensions, which allowed us to clone a 1.1kb fragment by PCR from a human cDNA library. Interestingly, the protein encoded by this highly regulated mRNA resembles that of novel F-Box protein (related to Fbx_{25}). Proteins containing F-Box motifs are a large family that function as substrate-binding subunits of E3s. Many F-box proteins are critical in the controlled degradation of regulatory proteins. Moreover, another of these unidentified atrophy-genes (AG-7) is a "ring-finger" protein, a motif characteristic of a catalytic subunit of most E3s. These exciting observations suggest that these two proteins are subunits of a new ubiquitination enzyme involved in the acceleration of protein breakdown during muscle wasting.

These results extend our prior findings on expression of proteasome genes, fit with our predictions about the changes likely to occur in fasting, and indicate a number of novel gene products that appear important in muscle wasting (including the potential discovery of a new highly-regulated E3 complex). Based on these encouraging results, a similar microarray analysis of rat muscles after hind-limb suspension has been initiated, and will be an important aspect of our future studies.

III. LIST OF PUBLICATIONS AND PRESENTATIONS SUPPORTED THROUGH NSBRI FUNDING

In total, 10 full articles were published in professional journals or monographs, and three are now in press that are based (in whole or part) on our studies supported by NSBRI. Eight of these are primary reports on research, and the rest are invited review articles on muscle protein degradation or proteasome inhibitors. In addition, 8 abstracts were presented at national or international meetings, and Dr. Goldberg gave a number of invited lectures covering this work.

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Effects of Unloading on Myosin Content and Isoform Specific Regulation in Skeletal Muscle

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Executive Summary.

History of the Project.

In the initial funding period supported by the NSBRI (October, 1997-September, 2000) our original project entitled "Effects of Unloading on Myosin Content and Isoform Specific Regulation in Skeletal Muscle" was jointly submitted along with a project proposed by Dr. Alfred Goldberg's group at Harvard Medical School (K.M. Baldwin and A. L. Goldberg, serving as Co-PIs). The central thrust of the proposal was to examine how unloading (and increased loading) states impact the expression of the MHC protein system by examining transcriptional, translational, and degradative (ubiquitin-proteasome) processes. Although the project was funded, the NSBRI appointed Dr. Goldberg as the PI and the proposal was given a new title with a primary focus on degradative processes associated with the ubiquitin-proteasome pathway. The Baldwin project had to be de-scoped, since the budget was reduced by > 50% thereby precluding performing any studies related to protein synthesis of the MHC isoforms due to insufficient resources. Instead our group focused on a narrower set of objectives: a) regulation of transcription of the slow MHC gene in response to altered loading states; and b) the delineation of myogenic factors in the control of muscle mass and contractile phenotype.

Progress on the Original Proposal.

Significant progress was attained on four interrelated projects: 1) in vivo regulation of the beta (slow) MHC gene in soleus muscle of suspended and weight-bearing rats; 2) changes in markers of myogenesis in overloaded rat muscles; 3) mechanisms on up regulation of fast IIb MHC in unloaded muscle; role of the nerve and thyroid hormone; and 4) quantitation of total MHC and MHC isoforms in response to unloading.

In the first project we demonstrated the feasibility of using direct DNA transfection technology for studies on the in vivo regulation of the type I MHC gene promoter in response to weight bearing activity and hindlimb suspension. In that project, we demonstrated that a) normal (optimal) type I MHC transcriptional activity in antigravity muscles requires the presence of an up-stream enhancer sequence (-3500 to –2900) that likely interacts with response elements in the first 400 bp upstream of the transcription start site (TSS); b) unloading-induced down regulation of type I MHC promoter activity is mediated in the proximal 400 bp upstream of the TSS. [We have tentatively identified the negative beta e1 response element as a key factor in this process]. Additional studies are in progress to more fully characterize the proximal response elements in response to unloading. A paper on this project has been published and is included in appendix A.

The second project was aimed at identifying markers of putative satellite cell proliferation and differentiation processes in muscles that undergo increases in hypertrophy due to increases in chronic loading. These experiments were performed in the context of increased expression of muscle IGF-I at both the mRNA and peptide level. The central findings of this study indicate that myogenic processes are activated in response to increased loading at early time points (e.g. 12 hrs) and that IGF-I is likely modulating this response. Furthermore, the findings indicated that some myogenic cells are likely differentiating early on in the adaptive process, before events leading to satellite cell proliferation have been initiated. Some of the data from this project is presented in the context of the current proposal in the next section. A copy of this paper is provided in Appendix A.

The third project was aimed at understanding how the ~ de novo expression of fast type IIb MHC gene occurs in antigravity muscles, e.g., muscle-types that do not normally express this gene. This work was predicated on the novel observation that hindlimb unloading requires increased levels of thyroid hormone in order to fully express the IIb MHC gene at both the mRNA and protein levels. Our finding suggest that normal innervation is essential for inducing the unique expression of the IIb MHC in a slow muscle in response to the combination of hindlimb suspension and thyroid hormone; and the up regulation of the myogenic factor, MyoD, may be essential to this process. However, in the denervated muscle, there is a discordance between the regulation of the endogenous IIb MHC gene relative to the exogenous IIb promoter-report construct that is not fully understood at the present time. A copy of the published article on these findings is provided in appendix A.

In the fourth project we developed techniques to quantitate changes in total as well as isoform specific MHC protein and mRNA content in response to unloading in order to show that during unloading, the myofibril system (and particularly the contractile apparatus) undergoes a remodeling in which there are reductions in the slow MHC content at the protein and mRNA levels which accompanies the general degradation process. In addition, there are also maintenance in protein and increase in mRNA content of fast MHCs (IIx-IIb) that occur in spite of the general atrophy process that predominates during unloading (see figure 2 in Section B). These findings, in conjunction with project III, clearly show that there is MHC isoform-specific gene regulation in response to altered loading states; and these processes are likely mediated by a coordination between transcriptional, translational, and degradation control points. We are in the process of writing a paper on this project, and have provided pertinent data on MHC content summarized in figure 2 the next section.

In summary, we have made significant progress on several fronts in an attempt to address fundamental issues in the biology of muscle plasticity that are relevant to the mission of the NSBRI. However, in view of the fact that future research concerning muscle structure and function funded by the NSBRI needs to be more closely related to seeking countermeasures for reducing muscle atrophy, we have refocused our research to more specifically address the efficacy and mechanisms concerning the role of resistance training in reducing the muscle atrophy that occurs in response to chronic unloading.

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PROJECT RESEARCH ACTIVITY

INTRODUCTION

During the course of evolving our research expertise to better address the mechanisms that regulate both muscle hypertrophy and atrophy in response to chronically altering the loading state imposed on skeletal muscle, our research team in year 3 of the funding period has begun to focus on the involvement of muscle-derived IGF–I gene expression as a pivotal player in controlling growth/atrophy processes in conjunction with the functional regulation of signaling pathways and key proteins that have been shown to play regulatory roles in controlling protein translation and protein degradation. Attention on the latter system, e.g., protein degradation is somewhat limited by design, because this area has been the primary focus of Dr. Goldberg's group. Hence, the derived information is being used for reference and context to those processes affecting translation. This prioritization of focus is being done in order to avoid duplication of research of other investigators in the NSBRI that focus more exclusively on the ubiquitin-proteasome system.

Background: Previous Findings and Theoretical Concepts

Recent work from our laboratory (figure 2) on the vastus intermedius (VI) muscle (a slow muscle that expresses predominantly type I MHC (~60%) along with 23% IIa, 13% IIx,

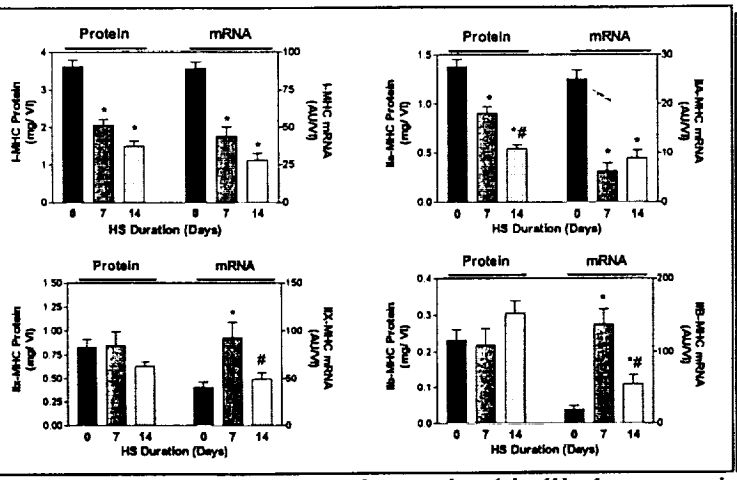


Figure 2:
VI MHC protein and mRNA isoforms content per muscle: Effects of 7 and 14 days of Hindlimb Suspension (HS). Columns are means, Bars are SEs; N=8/ea. Values were calculated based on muscle mass, myofibrils and total RNA yields, and % MHC Protein, and mRNA/18S signals obtained from 5μg of RNA on Northern Blots
VI Mass:
0d: 89±2; 7d: 74±4; 14d: 60±3

and 4% IIb MHC) has shown that hindlimb suspension (HS) causes a reduction in the amount of slow type I and fast type IIa MHC protein and mRNA expressed per muscle. In contrast, the amounts of IIx and IIb MHC protein and mRNA isoforms per muscle were either maintained or increased relative to the weight-bearing muscle. This response clearly illustrates the remodeling of contractile phenotype that takes place in atrophying antigravity muscles during unloading. This remodeling is the result of differential targeting of protein metabolism of slow vs. fast phenotype through mechanisms involving transcriptional, pretranslational, translational, and posttranslational (degradation) events. Currently, it is unclear what subcellular/molecular mechanisms are responsible for the observed changes in muscle mass and MHC protein isoform composition in response to unloading.

Molecular Mechanisms Controlling Protein Translation.

Protein translation is highly regulated with important control points clearly defined at three integrated levels: initiation; elongation; and termination. Of the three control points, available findings suggests that most of the control occurs chiefly at the level of initiation with additional control occurring at the level of elongation.

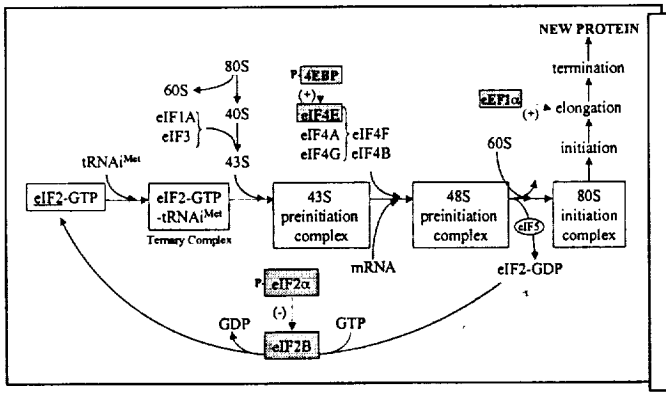


Figure 2.
mRNA Translation into Protein. Schematics of steps involved i protein synthesis. Shaded are factors thought to be critical in th regulation of the rate of protein translation in skeletal muscles. We are proposing to determine a) the phosphorylation states of eIF2α and 4EBP, b) the activity of eIF2B, and c) the expressio of eEF1α in order to evaluate the effectiveness of the resistanc training paradigm in opposing the unloading-induced ↓ protein synthesis.

Initiation of translation of most proteins is dependent upon two key ribosomal protein-mRNA binding mechanisms designated as a) the ternary binding process and b) the mRNA 5' cap-binding process. Changes in either the activity or capacity of these two processes have been suggested as the mechanism through which hormones (insulin; IGF-I), fasting/refeeding, nutritional states, and disease processes (sepsis/cancer) leading to muscle wasting regulate global protein synthesis.

The eukaryotic Initiation Factor 2 (eIF2) has been reported to play a central role in the maintenance of what is generally considered a rate-limiting step in mRNA translation. In this step, eIF2 binds GTP and Methionine initiator-tRNA and transfers the $tRNA_i^{Met}$ to the 40S-6S ribosomal subunit complex (figure 2&3). This represents the binding of the first amino acid to initiate protein synthesis. At the completion of this process, GTP bound to eIF2 is hydrolyzed to GDP by eIF5 and the eIF2-GDP complex is released from the ribosome. The exchange of GDP bound to eIF2 for GTP is a prerequisite to binding $tRNA_i^{Met}$, and this reaction is mediated by a second initiation factor, eIF2B, a complex protein composed of 5 subunits, α, β, γ, δ, and ε. The activity of eIF2B is a key control point for general protein synthesis regardless of the type of mRNA being translated (100, 131). Several mechanisms are involved with the regulation of eIF2B activity, including its own phosphorylation, phosphorylation of its substrate (eIF2), and by allosteric effectors. In what is probably the best-characterized mechanism for the regulation of mRNA translation, phosphorylation of eIF2 on its α-subunit converts eIF2 from a substrate of eIF2B into a competitive inhibitor. Thus, phosphorylation of eIF2α can prevent formation of the eIF2.GTP.Met-tRNA*i* ternary complex (figure 2) causing inhibition of global protein synthesis. Increased phosphorylation of eIF2α has been shown to occur under a variety of conditions including nutrient deprivation, insulin deficiency, and under certain stresses. In addition to eIF2α phosphorylation, eIF2B activity can be regulated by its own phosphorylation especially on the ε subunit. However the effect of this phosphorylation is variable, it could increase or decrease eIF2B activity depending on the cell system, and the kinase involved. Based on the above, it seems that tracking eIF2B exchange activity in muscle extract and regulation via its own (ε) phosphorylation or phosphorylation of eIF2α is important in assessing the effectiveness of any countermeasure paradigm in enhancing global protein synthesis. We hypothesize that resistance loading maintains dephosphorylation of the eIF2α, as well as enhances the enzymatic activity of eIF2B, likely via the action of IGF-I [a process similar to that of

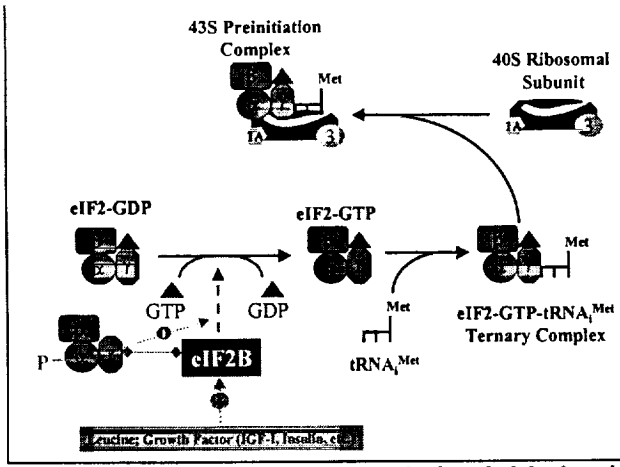


Figure 3.
eIF2B is a key factor in the initiation of protein synthesis. EIF2B mediates the exchange of GDP bound to eIF2 for GTP. eIF2 can bind tRNA$_i^{Met}$ only when bound to GTP, therefore the exchange of GDP for GTP mediated by eIF2B is a critical step in recruitment of the pre-initiation machinery. eIF2B is the rate limiting factor of this process, and its exchange activity is negatively regulated by phosphorylation of the α subunit of eIF2, which becomes a competitive inhibitor. Amino acids, insulin, and growth factors regulate eIF2B activity independently of the state of phosphorylation of eIF2α.

A second regulation step in translation initiation involves the binding of mRNA to the 43S preinitiation complex. This step is mediated by of a group of proteins (eIF4A, eIF4B, eIF4E, and eIF4G), collectively referred to as a complex called eukaryotic Initiation Factor 4F (eIF4F). During this step one subcomponent, e.g., eIF4E, binds to the m7 GTP cap structure (figure 6) located at the 5'-end of essentially all eukaryotic mRNAs and, through association of eIF4E with another protein, eIF4G, binding also occurs to the 40 S ribosomal subunit. The mRNA binding site is regulated through changes in eIF4E, with its phosphorylation state increasing a) its affinity for the cap

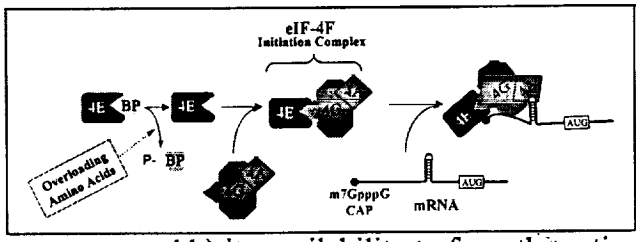


Figure 4. Schematics of the involvement of eIF4E in recruitment of the preinitiation factor eIF4F and mRNA before assembly into 48S (figure 4) in translation initiation. 4E is the only mRNA cap binding protein, and its abiliy to bind 4G depends on phosphorylation state of its binding protein (4EBP). Upon phosphorylation, BP is released making 4E readily available to incorporate into the eIF-4F preinitiation complex.

structure, and b) its availability to form the active eIF4E.eIF4G complex that is essential for initiation. Changes in eIF4E availability occur through modulation of the association of eIF4E with the translation repressor, 4 E binding Protein-1 (4EBP-1). eIF4E association with 4EBP-1, prevents the former from binding to eIF4G so that binding to the 43 S preinitiation complex is inhibited. Available evidence further suggests that factors activating the IGF-I receptor signaling pathway leading to the activation of the FRAP/mTOR kinase system (figure 5) cause subsequent phosphorylation of 4EBP-1. When 4EBP-1 becomes phosphorylated, it dissociates from eIF4E so that it can form the eIF4E.eIF4G complex necessary for initiation. The regulation of 4EBP-1 phosphorylation appears to be a critical step in the translation control of most cell systems including skeletal muscle. Studies show that protein synthesis activity in response to interventions such a insulin stimulation, feeding/fasting state, recovery from acute exercise, and amino acid (leucine) availability vary directly with the level of phosphorylation of 4EBP. As presented in more detail elsewhere in this proposal (see preliminary studies), we have obtained data to show that phosphorylation of 4EBP-1 is increased in response to functional overload (and acute resistance exercise) and this response correlates strongly with the level of IGF-I peptide induced in the overloaded muscle. Additional findings (see below) also suggest that exposure to acute episodes of hindlimb suspension transiently results in lower levels of 4EBP-1 phosphorylation, further suggesting a link between mechanical loading state and translational control at the level of initiation.

IGF-I Signaling Pathways and Regulation of Protein Synthesis.

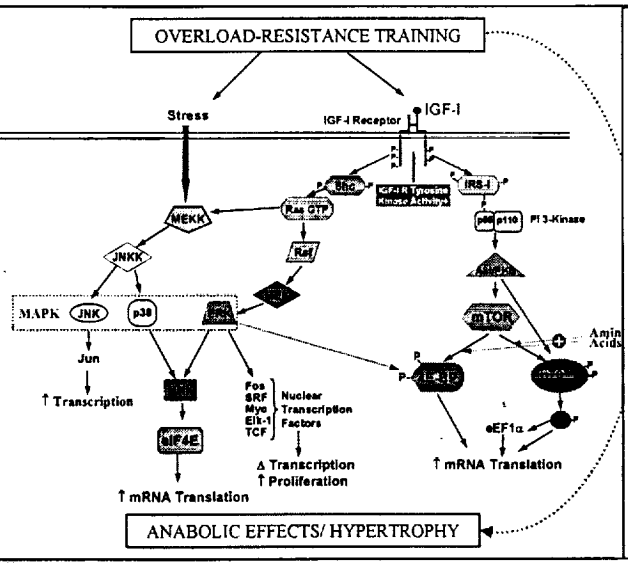


Figure 5. IGF-I signaling cascade. IGF-I receptor is a tyrosine kinase. Activation of IGF-I receptor by IGF-I leads its autophosphorylation and initiates a series of events dependent upon protein phosphorylation and protein-protein interactions to transmit the IGF-I signal from the receptor on the plasma membrane to intracellular compartments. Two parallel pathways are involved: one activating PI-3 kinase/mTOR, and the other activating ras/MAP kinase. Several key factors controlling protein translation are affected by this cascade. We're proposing to examine the expression and phosphorylation states of $P70^{S6K}$, ERK MAPK, and 4E-BP as a way to assess the effectiveness of the training program.

In previous sections we discussed the possible role of muscle produced IGF-I as a mediator linking mechanical activity to consequent muscle adaptation. Also we have discussed the important role that protein synthesis plays in the maintenance of muscle mass, and we presented information relevant to the molecular processes that control protein translation (figures 2,3,4). This section will discuss the regulation of protein synthesis by signaling pathways thought to be linked to growth factors such as IGF-I (figure 5). IGF-I anabolic action on skeletal muscle can be explained on the basis of the molecular signaling events initiated by its receptor, a tyrosine kinase activated upon IGF-I binding, and transmitted through a cascade of intracellular events involving phosphorylation/protein protein interaction of several regulatory proteins including some critical transcription factors and translation markers leading to a general increase in protein synthesis (figure 2).

We are proposing to examine the expression and phosphorylation states of two key kinases on this pathway: the $p70^{S6k}$ and the ERK-MAPK. The phosphorylation of $p70^{S6k}$ is mediated by the FRAP/mTOR kinase system, and has been associated with its regulation of subsequent phosphorylation states involving the S6 ribosomal subunit, as well as other initiation factors such as eIF4B and eIF4G. These responses are thought to play a significant role in translation control impacting specific groups of proteins. For example, increased phosphorylation of the S6 ribosomal protein has been associated with enhanced translation of a certain class of mRNAs that contain short (~8nt) polypyrimidine tracts (TOP) in their 5' untranslated region near their caps. These TOP mRNAs encode for ribosomal proteins and elongation factors such as eEF 1α and eEF2, which are involved in the regulation of cell cycle, proliferation and growth processes. In the context of these observations, recently, others have reported that resistance training of rat skeletal muscle caused muscle hypertrophy due primarily to increased protein translation activity that is mediated by increased phosphorylation state of the $P70^{S6k}$ protein. Furthermore, our research group shows that functional overload is associated with marked phosphorylation of the $p70^{S6k}$, and this response is highly correlated to the increased level of IGF-I induced by functional overload. Also, our initial studies (see below) show that 2-3 acute bouts of resistance training activate the level of IGF-I mRNA and protein, and phosphorylation state of $p70^{S6k}$ and of 4EBP-1, providing further evidence that mechanical loading provides a powerful stimulus to activate process

directly linked to increasing the rate of protein synthesis. The mTOR and ERK-MAPK phosphorylation will also be examined as other markers for activation of the IGF-I pathway; mTOR is located upstream of $p70^{S6k}$ and 4EBP-1 on the signaling pathway, while ERK is involved in regulating several transcription and protein translation factors (figure 5). Hence, we feel that the functional status of certain proteins can serve as useful cellular markers to assess the effectiveness of resistance training as a possible countermeasure to unloading-induced muscle atrophy.

Findings Supporting the Link Between Muscle Loading Conditions, IGF-I Expression, and IGF-I Signaling Pathways.

The data presented in this section were obtained from either recently published papers resulting from work accomplished in years 1 and 2, as well as recent experiments using either the functional overload model or 2-3 sessions of isometric resistance training in which the target muscles were induced to perform 60 contractions per training session. Each contraction was of 4-seconds in duration with a 16 sec rest interval between each contraction.

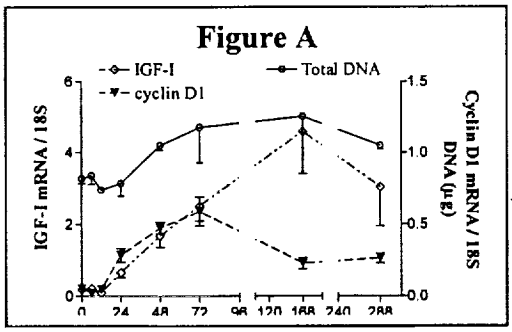
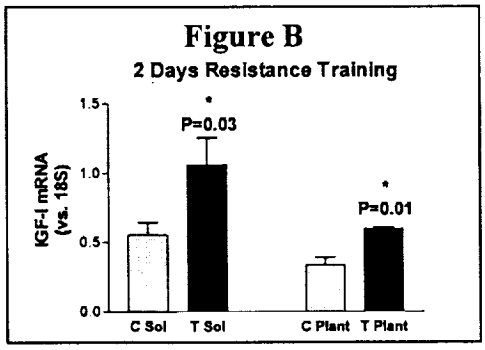


Figure A Effects of functional overloading on IGF-I muscle mRNA expression, and on markers for cell proliferation (DNA/Cyclin D1) in plantaris muscle. During functional overload, which imposes continuous stress during weight bearing on the overloaded, muscle IGF-I mRNA levels increase within 24h hrs. Both cyclin D1 (a cell proliferation marker) and DNA concentration increase by 24-48 hours following overload. This suggests that mechanical stress, leading to net protein accumulation and hypertrophy, activates cell-cycle markers consistent with myogenic proliferation of satellite cells.



Up regulation of the IGF-I mRNA signal was also seen in both soleus (Sol) and plantaris (Plant) muscles after two training sessions involving resistance training (**figure B**). C: Contralateral control, T: Trained

Existing evidence supports the role of IGF-I in mediating satellite cells proliferation and differentiation into muscle cells. The involvement of satellite cells in muscle growth and adaptation in response to overload is further supported by our recent finding that gamma irradiated muscles fail to hypertrophy to compensate for the overload stimulus (**figure C**). Gamma irradiation destroys the satellite cells ability to proliferate.

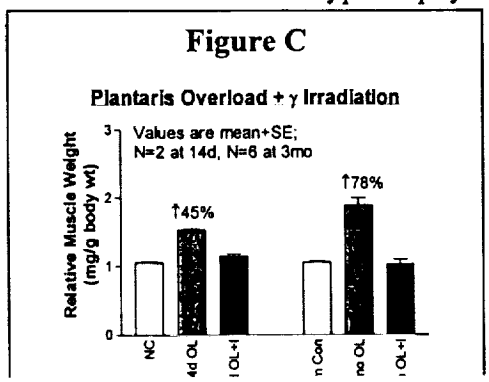
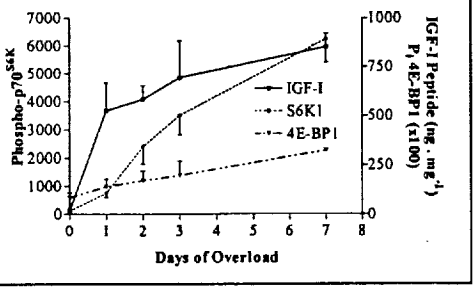


Figure C: Gamma Irradiation Experiment: A group of 8 rats were anesthetized, gamma irradiated on the left lower limb while the rest of

the body was protected from the radiation. On the next day, the plantaris muscles on both left and right legs were overloaded with surgical removal of synergists. 2 rats were killed after 14 days from the overload, and their muscles were weighed; while the remaining six rats were kept in their cages under normal activity for a total of 3 months (90 days), at which time they were killed and their muscles were weighed. Data in figure C show that the relative muscle weight to body weight of the overloaded non irradiated plantaris increased by 45% and 78% by 14 d and 3 mo respectively, while the irradiated plantaris relative weight did not increase over the control at both time points.

Figure D



Effects of Overload on Markers of Protein Initiation. In **figure D,** we present data showing the temporal relationship in overloaded plantaris between muscle IGF-I expression and phosphorylation state of p70 S6 kinase and the 4EBP-1, markers of key proteins involved in increasing translation processes. Note the strong correlation between IGF-I expression and phosphorylation status of the two markers. Consistent with the protein accumulation and the cell proliferation markers presented above, these results strongly suggest that these proteins may serve as a useful marker for determining the status of a muscle undergoing positive protein balance in response to loading stimuli.

Expression of a Muscle-Specific IGF-1 mRNA Variant Termed Mechano-Growth Factor(MGF).

With the recent emerging information on the importance of muscle produced IGF-I isoform and mechanotransduction mechanisms, we designed PCR primers to specifically amplify the muscle specific IGF-I isoform, MGF. We analyzed MGF mRNA expression in both soleus overload (after 2, 4, and 6 days), and resistance training (3 training sessions); both models were associated with an increased expression of the MGF-I in the target muscle (figure E). A similar pattern of expression was also shown in the plantaris muscle (data not shown).

Figure E

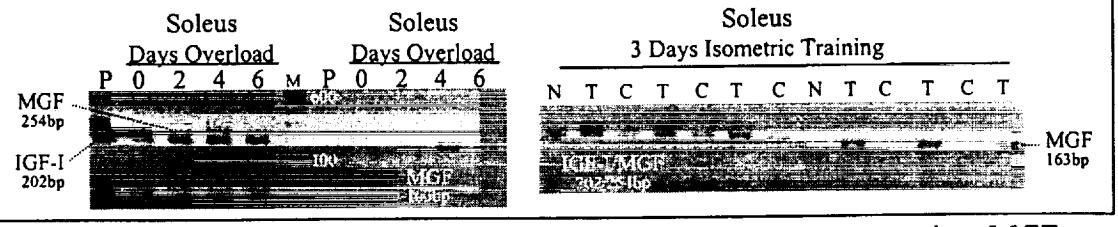
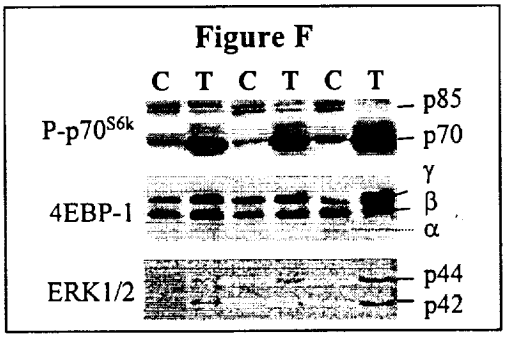


Figure E: In the RT-PCR reaction used to determine IGF-I mRNA expression, MGF (IGF-I- variant) product appears as 52 bp larger than the major band (IGF-I). We designed new primers which detect only MGF mRNA; the product of this reaction is 163bp. Note the increase in both IGF-I and MGF to mechanical stimuli; In fact, MGF was not detectable (under the test condition) in a normal control muscle. P: normal control Plantaris, N: Normal control soleus, C: Contralateral control, T: Trained, M: molecular weight markers (100bp ladder, Gibco BRL).

Markers of Protein Translation in Resistance Loaded Muscles.

Since resistance training is likely to be a primary modality in attenuating the atrophy that occurs in response to prolonged spaceflight, our initial goal is to determine what effects acute bouts of resistance loading has on protein translational processes in skeletal muscle. We are currently focusing on three markers as delineated in Figure F.



Figures F: Markers of protein translation in soleus muscles in response to acute isometric resistance training (3 training sessions). Note the same directional changes in pp70S6k and 4EBP-1, and ERK1/2 phosphorylation state. These markers were analyzed by immunoblotting techniques using phosphospecific antibody angainst $p70^{S6k}$, antibody against 4EBP recognizing different migrating forms on the gel reflecting the different phosphorylation states, and anti phospho specific ERK Map kinase which recognize two forms: p42 and p44. The same pattern of expression was also observed in the plantaris muscle (data not shown).

Effect of Acute Resistance Training On A Negative Regulator of Protein Initiation Processes.

When eukaroytic Initiation Factor 2α (eIF2α) undergoes phosphorlylation it has been reported to serve as a negative modulator of a rate-limiting step in protein initiation called ternary complex formation. Thus, we are studying how phosphorlyation of this protein is affected by resistance loading states (see figure G).

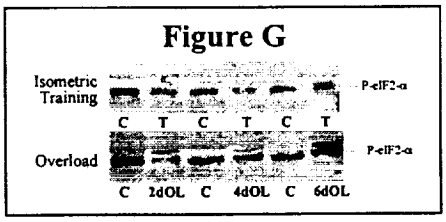
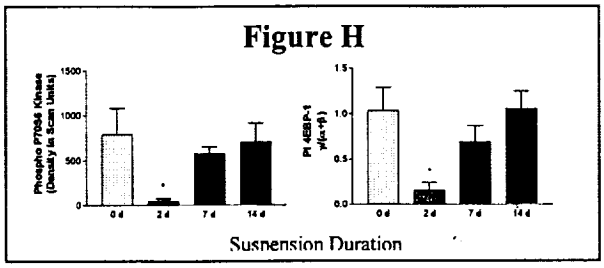


Figure G Both resistance training and overloading tend to decrease **eIF2α phosphorylation** in the plantaris, a fast-twitch muscle

C: contralateral control; T: 3-day isometric resistance trained; OL: overloaded (2d: 2 days; 4d: 4 days; and 6d: 6 days)

Effects of Short Term Hindlimb Unloading onTranslation Markers.

In the above sections we provided evidence that phosphorylation state of the protein markers for protein translation were increased in response to increased loading. The Data presented in figure H suggest that the opposite occurs when muscles are subjected to unloading.



$P70^{S6k}$ and 4EBP data **(figure H)** show an abrupt decrease of the phosphorylation states of these two translation markers in the soleus muscle at 2 days after hindlimb suspension, however, this is partially recovered at 7 days of unloading. This observation may indicate that protein synthesis efficiency needs to be back to normal to achieve synthesis of new protein involved in the remodeling of the unloaded slow muscle to a faster phenotype. We are proposing to measure the activity of eIF2B initiation factor as this factor could be more closely related to global protein synthesis. Also, we are in the process of a) examining the phosphorylation state of eIF2 α subunit, which correlates with inhibition of eIF2B activity, and b) determining the expression of elongation factor 1α, which could be limited under unloading condition.

Data Relevant to Protein Degradational Processes.

Dr. Goldberg's group has provided strong evidence that the ubiquitin-proteasome pathway is a central player in causing net protein loss in muscle in response to a variety of states that result in muscle wasting, including hindlimb unloading. Consistent with these findings we have shown that one of the key enzymes in the ubiquitination of target proteins involves the E2 14k Ub carrier enzyme. Results show that mRNA encoding this enzyme is sensitive to loading state being decreased in expression during increased loading and increased under conditions in which the muscle is unloaded.

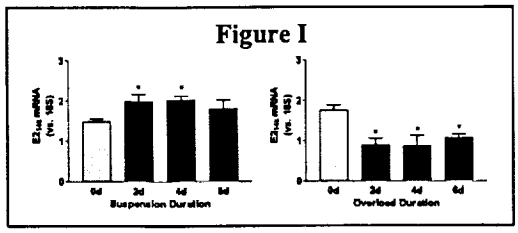


Figure I. <u>E2 14k Ub carrier enzyme mRNA, response to loading condition in the soleus.</u> E2 14k mRNA was determined using the relative RT-PCR method (Ambion), in which the target RNA cDNA is coamplified with 18S ribosomal RNA using specific primers for each. This enzyme of the Ub-proteasome degradation system is thought to be involved in the regulation of the rate of muscle protein degradation.

Effects of Loading on Enzymes Regulating Sarcomeric Protein Turnover.

Proteins that make up the myofibrillar fraction of skeletal muscle, e.g., so called sarcomeric proteins that form the contractile machinery, undergo constant turnover with a half-life of several days. One of the proteolytic systems thought to be involved in the degradation of the myofibrils for presentation to the Ub-proteasome system is the calpain family. We have begun experiments to examine their expression under aletered loading states. Our preliminary findings (Figure J) suggest that calpain I has lower levels of protein expression in muscles that are subjected to stimuli that result in increased accumulation of myofibril protein. These findings suggest that when muscles are loaded they may have a lower rate of myofibril turnover thereby favoring greater net accumulation of protein. Further, the findings suggest that loading may slow degradative processes by decreasing the expression of enzymes affecting net protein degradation.

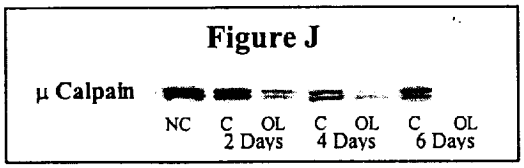


Figure J. Calpain I (μ Calpain) detection in overloaded soleus muscle. Calpain was detected by chemiluminescence (ECL) after immunoblotting using an antibody form Calbiochem, note the notable decrease with overload.

Implications For Future Research Directions.

The above data provide ample evidence that isometric resistance training is associated with increased muscle expression of IGF-I (MGF), as well as signaling pathways and marker proteins previously shown to be involved in enhanced protein synthesis and muscle growth. Based on these observations our goal is to define optimal conditions for maintaining protein translational processes at levels sufficient to attenuation the atrophy that is associated with chronic unloading. Our working hypothesis is that when skeletal muscles become chronically unloaded, as occurs during spaceflight, the activity levels (functional state) of enzymes controlling protein translation are insufficient to counteract the processes that control protein degradational process and hence there is net loss in protein mass in the affected fibers. Data supporting this concept have been presented in the above sections. The goal for the future is to examine how programs of resistance training affect the above processes when the training regimens are performed in the context of stimuli such as hindlimb unloading. The goal is to evolve a prescription in which resistance loading stimuli are presented in sufficient quantity and frequency in order to attenuate the dominance of protein degradation relative to protein translation in order to maintain muscle protein balance in the muscles.

Appendix A: List of Publications

1. Adams, G. R. , F. Haddad, and **K. M. Baldwin**. The time course of changes in markers of myogenesis in overloaded rat skeletal muscles. J. Appl. Physiol. 87: 1705-1712, 1999.

2. DiMaso, N. A., F. Haddad, M. Zeng, S. s. McCue, and **K. M. Baldwin**. Role of denervation in modulating IIb MHC gene expression in response to T3 plus unloading state. J. Appl. Physiol. 88:682-689, 2000.

3. Giger, J. M., F. Haddad, A. X. Qin. And **K. M. Baldwin**. In vivo regulation of the β myosin heavy chain gene examined in soleus muscle of suspended and weight-bearing rats. Am J. Physiol. Cell Physiol. 278: 1153-1161. 2000.

FINAL PROGRESS REPORT

NOVEMBER, 2000

MOLECULAR SIGNALING AND ASSEMBLY IN MUSCLE PLASTICITY

HENRY F. EPSTEIN, M.D.

MUSCLE ALTERATIONS AND ATROPHY TEAM

NSBRI

A. Original Specific Aims:

1. Identifying the roles of dystrophin-based pathway as a signaling pathway for muscle plasticity most active in Type II fibers. The *mdx* null mouse line which is dystrophin-negative will be studied.
2. Delineating the roles of the myotonic protein kinase (DMPK)-based system which appears to represent a signaling pathway for muscle plasticity most active in Type I fibers. Knockout and transgenic mouse lines which are either DMPK-negative or overexpressing DMPK will be studied.
3. Characterizing the roles of focal adhesion kinase and associated molecules as a general myogenic signaling pathway in muscle plasticity. Transgenic mouse lines with inducible knockout and mutant constructs will be studied.
4. Analyzing genetically the interactions between dystrophin, focal adhesion kinase, and DMPK pathways in specific double mutant combinations, and further characterizing the mechanisms by which those pathways regulate muscle plasticity.

Because of restrictions in funding from NSBRI and the lack of matching funds from NSBRI contacts, we have concentrated our efforts on Specific Aim 2 and closely related aspects of Specific Aims 3 and 4. The central molecule of interest has been DMPK. The interactions of DMPK with Rac-1 that we have studied are related *in vivo* to the actions of focal adhesion kinase. Rac-1 is a downstream effector of focal adhesion kinase which is an integral component of the integrin/actin cytoskeleton signaling pathway that is adhesion-sensitive. The interactions of DMPK with Raf-1 kinase of the chemical signaling pathway and Rac-1 represent an important further characterization of the signaling pathways which regulate muscle plasticity.

In the last year, our work on the UNC-45 myosin assemblase has become relevant to this project because we broadened our focus there from genetic and biochemical experiments in *C. elegans* to the identification and characterization of UNC-45 homologues in humans and mice. We will discuss Progress in our laboratory from the perspective of the two molecules of central interest: DMPK and UNC-45.

B. Progress Report

DMPK and Cross-talk: DMPK is a multi-functional enzyme with several distinct regions of structural and functional significance (1, copy appended) (Figure 1). The L domain is a single leucine-rich motif (2). ThePK domain is the canonical serine-threonine domain for the myotonic dystrophy kinase family (3). The H domain is an α-helical, coiled coil domain that is also present in many members of this kinase family (4). The T domain resembles transmembrane domains of other enzymes that associate with the cytosolic face of the endoplasmic reticulum. Both the L and H regions are likely to be responsible for binding protein partners that either regulate DMPK or further restrict its localization. The H region shows homology to known Rho GTPase binding sites whereas the L region has potential interactions with beta-tubulin by yeast two hybrid complementation (our unpublished results). Several members of the DMPK family are known RhoA protein kinases.

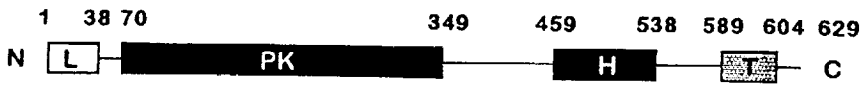


Figure 1: DMPK substructure

We have, therefore, studied the interaction of DMPK with members of the Rho family of GTPases (5). Both RhoA and Rac-1 could physically bind DMPK in contrast to cdc42 and Ras. Rac-1 showed the greatest affinity. In COS-M6 cells, cotransfection of activated Rac-1 led to an almost 3 fold enhancement of DMPK's transphosphorylation of histone H1 (Figure 2).

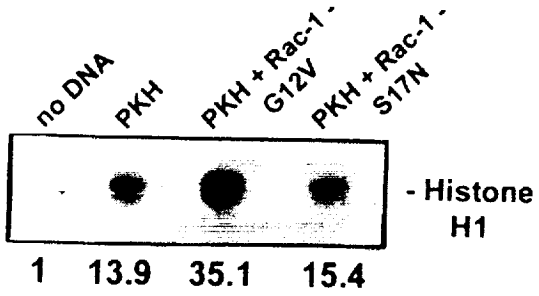


Figure 2: Constitutively activated G12 Rac-1 leads to over two-fold activation over constitutively inactive S17 Rac-1 or PKH alone.

Inactive Rac-1 showed no activation of DMPK. Overexpression of DMPK, RhoA, or Rac-1 in human lens epithelial cells all lead to reorganization of the actin cytoskeleton and membrane blebbing (6, copy appended). These results are consistent with DMPK having a specific, functional role along with Rac-1 and RhoA in the regulation of the actin cytoskeleton.

Raf-1 kinase, a key component of Ras-activated MAP kinase signaling pathway clearly phosphorylated DMPK. The catalytically inactive DMPK K100A mutant was phosphorylated by activated Raf-1 kinase (5, copy appended). When active DMPK (LPK) and Raf-1 kinase are incubated together, the phosphorylation of both and of histone substrate is

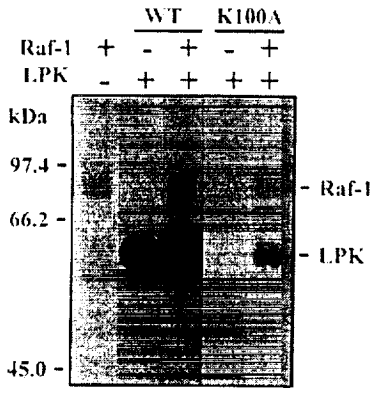


Figure 3: Raf-1 kinase phosphorylates LPK.

enhanced (Figure 3). Yeast two-hybrid complementation verified that DMPK and Raf-1 kinase can physically interact with one another.

Therefore, we have proposed that DMPK may be a point for cross-talk between the integrin/actin cytoskeleton-based signaling pathway represented by Rac-1 and the growth factor receptor/Ras/MAP kinase signaling pathway represented by Raf-1 kinase (Figure 4).

DMPK and Muscle Plasticity: This work focuses on studies with knockout mice (7, 8). The absence of DMPK appears to have an effect on the differentiation of specific muscle fiber types as well as leading to an altered response to unloading in mouse soleus. In these experiments, we have compared wild type (+/+), heterozygotes (-/+), and homozygous knockouts (-/-) from the same litters. All animals were 3 month old females. This practice minimizes the effects of genetic background other than the gene of interest. Soleus muscles from wild-type and heterozygote mice were indistinguishable in either control or tail suspension experiments in terms of their muscle fiber type distribution as monitored by myosin heavy chain characterization using a specialized modification of SDS/PAGE (9) (Figure 5). Type I myosin became reduced in both genetic forms with unloading and Type II myosins, particularly Type IIB, were elevated. In contrast, the homozygous knockouts showed elevated Type IIB in the control situation, as if the absence of DMPK created an unloaded-like state. Unloading produced abnormal changes in the relative amounts of Type IIA and IID/X when compared to wild-type and heterozygous mice. These experiments suggest that DMPK must play a significant role in the modulation of skeletal muscle differentiation into specific fiber types and its alteration in response to unloading.

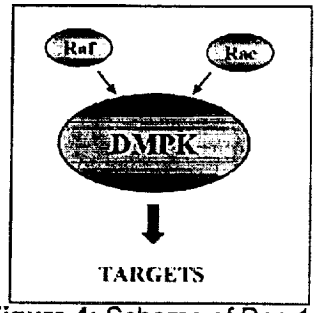


Figure 4: Scheme of Rac-1 and Raf-1 kinase cross-talk at DMPK.

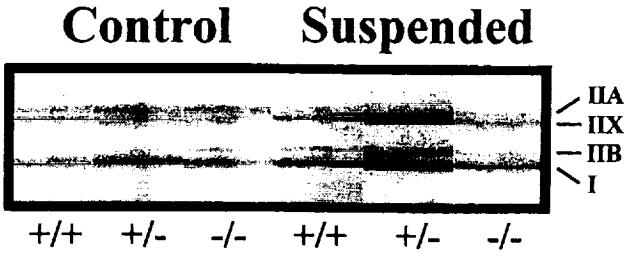


Figure 5: SDS-PAGE of myosin heavy chains from soleus muscle extracts of distinct murine lines under free-moving and tail suspension.

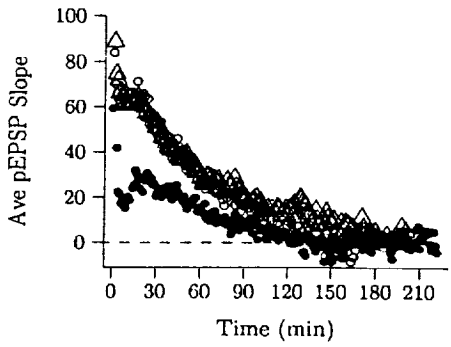


Figure 6: Intermediate-term potentiation (ITP) of the hippocampal CA1 region is selectively decreased in homozygous knockout (-/-) but not heterozygous (-/+) wild-type (+/+) mice.

We have used monoclonal antibodies specific to Type I myosin (BA-D5) and to Type II myosin heavy chains (SC-75) (10) to verify the SDS/PAGE analysis in two ways. First, we have used them in immuno-histochemical analyses of frozen sections of soleus muscles of control or suspended mice with each genotype. Second, we have performed Western blots of SDS/PAGE of each of these states. Our preliminary results here are consistent with the hypothesis that DMPK plays an important role in skeletal muscle differentiation into specific fiber types, their plasticity in response to alterations in loading and the expression of their major protein specific myosin heavy chain isoforms.

DMPK and Synaptic Plasticity: Parallel studies to our muscle work have been performed on synaptic plasticity in the hippocampus in collaboration with Dr. Paul Schulz of the Department of Neurology at Baylor College of Medicine. The hippocampal formation of the brain cerebral cortex is necessary for learning and short-term memory. DM patients show deficits ranging from decision making to significant mental retardation. Dr. Schulz has been able to resolve the response of the hippocampal CA1 neurons to tetanic stimulation into three processes, or STP (short-term potentiation), ITP (intermediate-term

potentiation), and LTP (long-term potentiation). Various drugs that inhibit STP or LTP do not affect ITP. Mice of all three genotypes with respect to DMPK (+/+, -/+, -/-) were examined with respect to these plastic responses to tetany. Male 3 month old littermates were compared. There is a specific 63% decrement of ITP in homozygous knockouts but not in the heterozygous or wild-type mice (Figure 6). The normal physiology of the CA1 neurons and their STP and LTP were normal in all genotypes. These results represent the first experimental finding related to DMPK function in the central nervous system. They also link a newly identified process in synaptic plasticity, potentially relevant to learning and memory, ITP, to a specific molecule, DMPK. This work raises the possibility that part or all of the altered muscle plasticity in DMPK knockouts may also be an example of synaptic plasticity, that of the neuromuscular junction. A separate collaboration with Dr.Ashok Balasubramanyam of the Department of Medicine has localized DMPK to the hippocampal formation by immunocytochemistry (11, copy appended).

New DMPK Antibody: We have recently obtained rabbit antiserums to recombinant DMPK from the laboratory of Dr. Charles Thornton at the University of Rochester School of Medicine. As Figure 7 shows, these antibodies show a marked reduction or absence of reaction to soleus muscle Western blots of the DMPK knockouts versus wild-type. Further tests of different tissues, bleeds, and antibodies purified by affinity chromatography against recombinant DMPK are in progress. If all tests for monospecificity to DMPK are met, this antibody will be a powerful reagent for our proposed work.

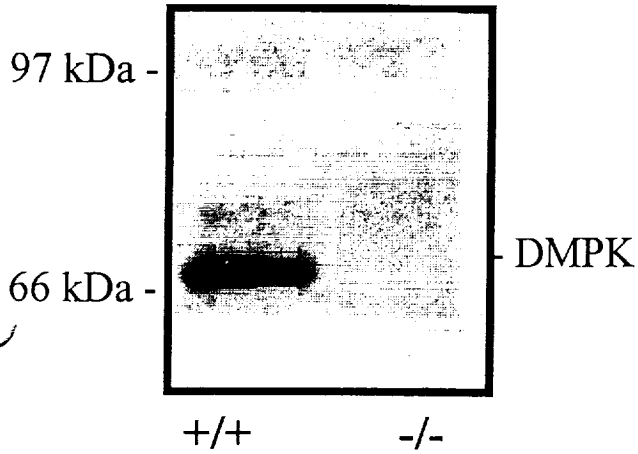


Figure 7: Antiserum FP8 shows reactivity with a single major band at the expected M_R of full length LPKHT in Western blot of extracts of soleus muscles from wild-type (+/+) but not DMPK knockout (-/-) mice. The higher minor specific band may represent phosphorylated LPKHT.

UNC-45 As a Myosin Assemblase: The first characterization of the actin of the *unc-45* gene was through the discovery by me of a temperature-sensitive mutation affecting body-wall muscle organization and contractility in *C. elegans* many years ago (12). The mutants when grown at 15° C resembled wild-type, but when grown at 25° C, they became severely paralyzed and the myofilaments were severely disorganized and appeared reduced in number. Most significantly, reversal of temperature during embryonic or larval development led to reversal of phenotype. However, once reaching adulthood, phenotype was not reversed by changing temperature. This result suggested that some process required for or in filament assembly during development was being affected.

Our interest in this work resumed when the sequence of the *unc-45* gene was accomplished (13) and verified at the cDNA level by us (14). Analysis of the predicted 961 amino acid sequence revealed two regions with similarities to previously identified proteins: the amino terminal TPR domain and the carboxyl terminal UCS (coined by us) region (Figure 8). The TPR domain contains three TPR (tetratricopeptide repeats of 34 amino acids each) motifs which closely resemble similar sequences in many signal transduction proteins, protein foldases, molecular chaperones, and co-chaperones. The UCS (standing for Unc-45, CRO1, She4p, described in background) region is closely related by amino acid sequence to the products of the previously genetically identified CRO1 and She4p fungal genes. In fact, all of the known *unc-45* mutations map in or closely to this region (temperature-sensitive) or would prevent its expression (stop codons) (Figure 8). The sites of the temperature-sensitive mutations are all conserved between the three species (and also now human, mouse, zebra fish, and *Drosophila*), and indeed the *S. pombe* mutations are identical to some of the *C. elegans* ones.



Figure 8: Scheme of UNC-45 substructure.

A major breakthrough in studying the mechanism of UNC-45 was its successful expression in baculovirus-infected Sf9 cells by Jose Barral. Soluble, monomeric, native protein was highly purified (> 95%) by nickel affinity chromatography (through an amino-terminal polyHis tag), monoQ anion exchange chromatography and Superdex gel filtration chromatography. Although the predicted polypeptide MW is 109,000, UNC-45 moved on Superdex as a 183,000 MW species, consistent with it being an anisotropic or markedly ellipsoidal monomeric molecule. Limited incubation with either chymotrypsin or trypsin led to a single cleavage (the two cleavage sites are two residues apart) in the middle of the UCS domain. Amino 70,000 and a carboxyl 39,000 dalton fragments were produced, but remain associated with one another. These results are highly consistent with the UNC-45 preparation being native, and with UNC-45 molecules containing a flexible hinge or loop between closely interacting 70 and 39 kDa segments. Quick freezing of purified UNC-45 permitted long-term storage with retention of the native molecular weight and cleavage properties.

This UNC-45 preparation bound both HSP90 and myosin form *C. elegans*. A truncated UNC-45 protein without the TPR domain did not bind HSP90 under the conditions that the full-length bound (Figure 9). HSP90 and UNC-45 protein formed a stable 1:1 complex that migrates at a higher MW on Superdex than either molecule itself. Myosin in larval homogenates was bound by UNC-45 protein immobilized through a FLAG epitope engineered at its carboxyl terminal (Figure 10).

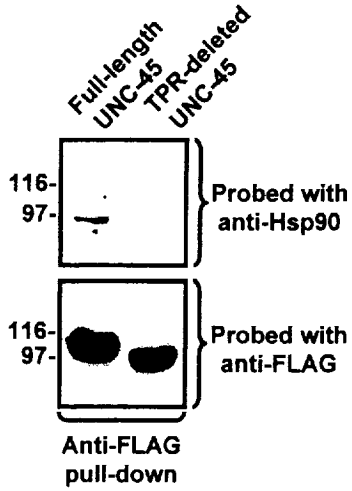


Figure 9: UNC-45 requires TPR domain for HSP90 binding. Western blot was developed by chemiluminescence.

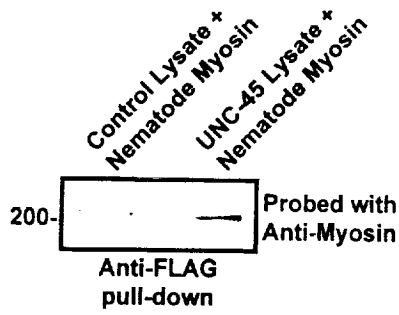


Figure 10: UNC-45 binds myosin: Western blot was developed with monoclonal anti-*unc-54* myosin heavy chain and detected by chemiluminescence.

Human and Mouse UNC-45: Computer searches for homologues to the *C. elegans* UCS region by Dr. Maureen Price revealed related sequences in human, mouse, zebra fish, and *Drosophila* genomic or cDNA sequence databases. A probe for the first human UNC-45 sequence detected localized to human chromosome 15q25-26. This corresponding genomic DNA has been cloned and sequenced. From cDNA databases, she was able to reconstruct the coding region, and its predicted amino acid sequence is about 40% identical to the entire sequence of *C. elegans* UNC-45. These cDNAs represented a variety of different tissues, and we provisionally consider this gene and protein to be related to general cytoskeletal functions. Mouse homologues were found to the human and *C. elegans* sequences. These were pieced together from cDNAs. The homologue closest to the first human sequence was obtained from pooled organ but not muscle cDNAs, consistent with it representing a cytoskeletal protein. A second set of cDNAs from a mouse myotube cDNA library revealed highly similar but distinct cDNA and predicted amino acid sequences near the 3' and carboxyl terminals. We provisionally consider this sequence to represent a sarcomeric form of UNC-45. The second mouse cDNA sequence permitted us to identify a second human sequence. The predicted partial amino acid sequences for all four mammalian UNC-45 proteins; human cytoskeletal, mouse cytoskeletal, mouse sarcomeric, and human sarcomeric are presented in Figure 11. The corresponding chromosome locations (as presently determined) are 15q25-26, 7, 11, and 17, respectively. Interestingly, there is a cluster of sarcomeric myosin heavy chain genes on 17.

Mammalian UNC-45 Homologues

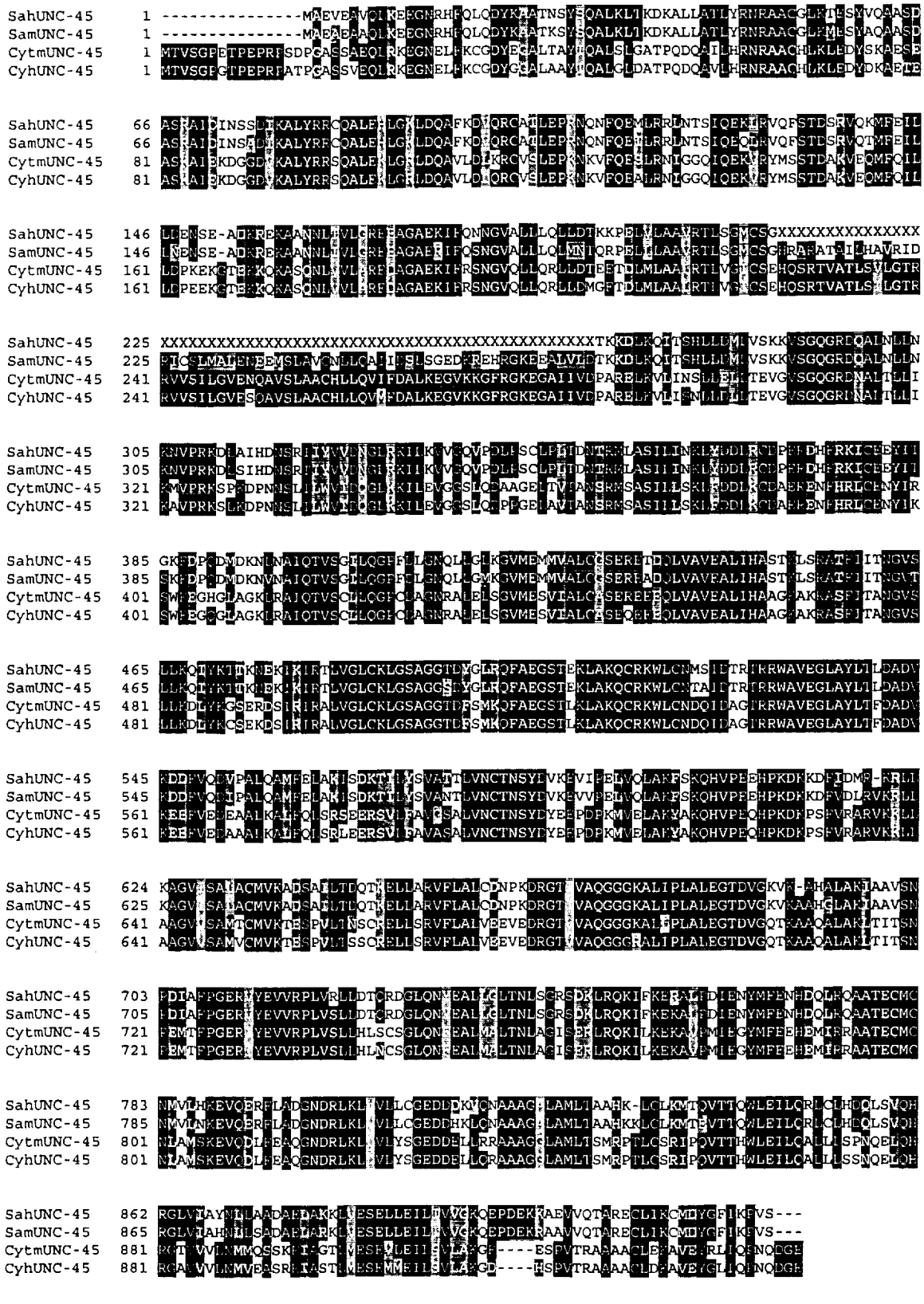


Figure 11: Amino acid sequences of mammalian UNC-45 protein. Only the human cytoskeletal isoform has its complete sequence known by genomic sequencing. The completion of the other sequences is in progress.

Dr. Price has taken specific cDNA probes for mouse cytoskeletal and sarcomeric UNC-45 and studied their expression by *in situ* hybridization of mouse embryos and by Northern blots of RNAs from multiple adult mouse organs. The cytoskeletal and sarcomeric designations have been confirmed by the embryonic expression patterns. The cytoskeletal probe labeled many sites, particularly analogs of organs or limb buds as they begin proliferation and very early differentiation. The sarcomeric probe labeled the developing heart by 8.5 days and somites by 9 days (Figure 12).



Figure 12: In situ hybridization of sarcomeric probe to 9 day mouse embryo. Note prominence of heart and somites.

Northern blots of adult mouse RNAs show that the cytoskeletal probe reacted with the 3.35 kb RNA of multiple organs. Whereas the sarcomeric probe reacted very strongly with the 3.12 kb RNA of skeletal muscle and heart. Very interestingly, the sarcomeric probe reacted more weakly with the lung 2.8 kb RNA species (Figure 13). We do not know whether this third species is a spliceoform of the sarcomeric gene or possibly, the product of a third gene.

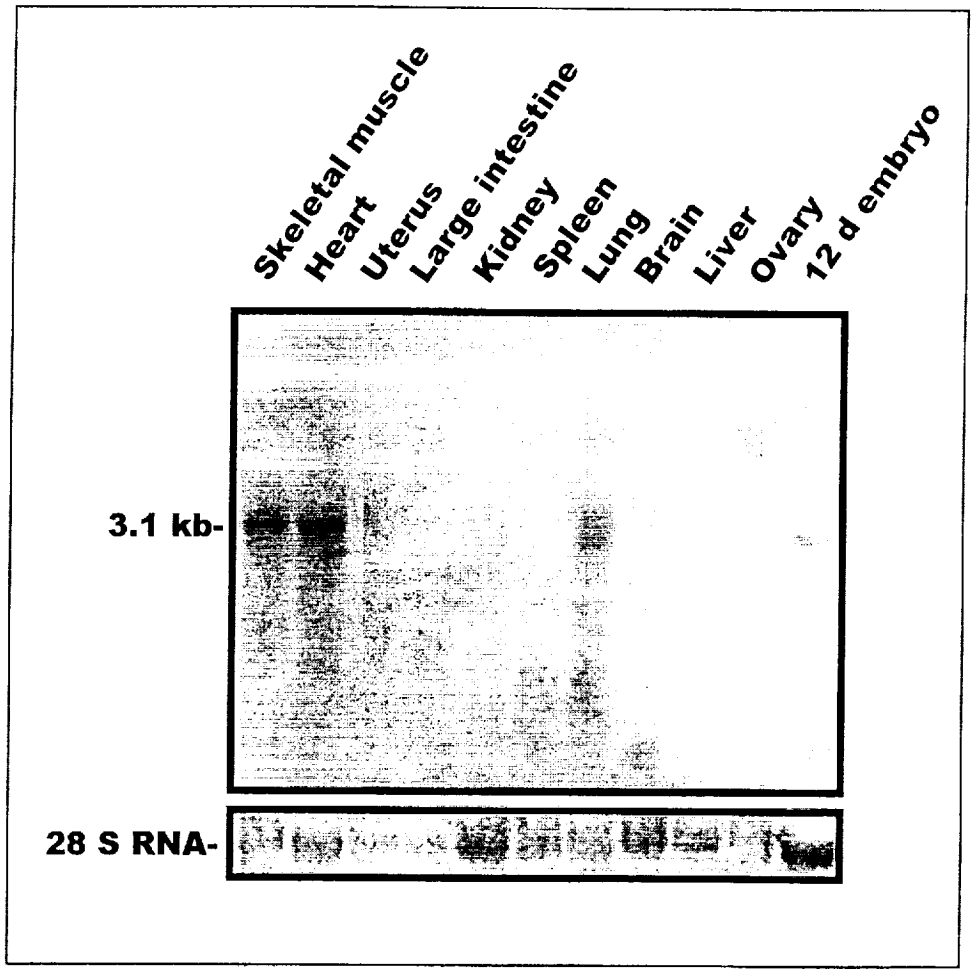


Figure 13: Northern blot of multiple adult murine tissues with sarcomeric UNC-45 3' cDNA probe. Note specificity of heart and skeletal muscle.

The finding of at least two genes for UNC-45 in both mouse and human should permit us to dissect their separate functions. The questions raised by the general cytological functions of the fungal UCS proteins and the specific muscle-defective phenotype of *C. elegans unc-45* mutants may now be answered through mouse genetics and biology. Interestingly, both *C. elegans* and *Drosophila* appear to have single UNC-45-related loci on chromosomes III and 3, respectively. The genetics of *unc-45* in *C. elegans* may not reveal its more general, early embryonic functions because maternal UNC-45 mRNA may rescue a loss-of-function as has been proposed for mutations affecting its HSP90 partner that produce only larval-defective phenotypes. *Drosophila* may present a similar problem. In this case, the different developmental mechanisms of mice versus worms or flies may permit us to resolve these questions.

Publications (1998 – 2000)

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Activity Dependent Signal Transduction in Skeletal Muscle

Hamilton, S.L.

This Project Report is not included.

Molecular Mechanisms Regulating Muscle Fiber Composition Under Microgravity

Rosenthal, N.A.

This Project Report is not included.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Project Report
October 2000

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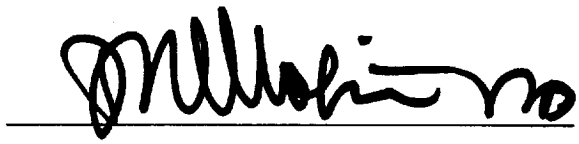
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Motoneuron Influences on Muscle Atrophy in Simulated Microgravity

EXECUTIVE SUMMARY

Many alterations in motor unit structure and function occur with exposure to microgravity during spaceflight, and could impair motor performance. While much work is ongoing to ascertain the nature of biochemical, structural, and physiological changes occurring in muscle fibers, little attention has been paid to the changes reported in motoneuron terminals at the skeletal neuromuscular junction, and in motoneuron cell bodies, during exposure to microgravity. It is unlikely that these changes, whether they occur independently or secondary to changes in the innervated muscle fibers, are without consequences for the regulation of motor unit function. Accordingly, **the central hypothesis of this study is that alterations in motoneuron structure and function occur during the process of microgravity-induced muscle atrophy, and that these alterations significantly influence muscle dysfunction, adaptation, and recovery from atrophy induced by microgravity**. These changes may be manifested as early structural and functional alterations in the distal motoneuron terminal, in addition to alterations in motoneuron activity produced by changes in stretch reflexes and supraspinal pathways. Initiation of alterations in motoneuron terminals may be influenced by retrograde signals from muscle which induce, as an early event, changes in intracellular calcium and transmitter release. To begin to address these hypotheses, a combination of electrophysiologic assays of transmitter release at neuro-muscular junctions, coupled with electron microscopic assays of junctional remodelling, synaptic vesicles, and intraterminal calcium, is being used to define quantitatively the nature, extent, and possible significance of changes in motoneuron terminals occurring in a mouse model of unloading-induced muscle atrophy.

During the course of this work, a technique for S-SFEMG (stimulated single-fiber electromyography) was adapted and validated for mice, allowing *in vivo* measurements of neuromuscular transmission. Data from this work suggest that: (1) unloading of skeletal muscle is associated with altered transmitter release at neuromuscular junctions, ultrastructural abnormalities, and a reduced safety factor suggesting insecure neuromuscular transmission; (2) the extent and nature of these junctional alterations vary among individual hindlimb muscles, possibly relating to differences in muscle loading and/or fiber type composition; (3) the extent of junctional alterations varies with duration of hindlimb unloading; (4) unloading of skeletal muscle may be associated with increased calcium in the motoneuron terminal, which may act as a signal inducing the observed junctional alterations; and (5) altered neuromuscular junctions in unloaded muscle retain the insensitivity to acute muscle stretch typical of normal mammalian junctions. Based on our observations, we hypothesize that junctional remodeling associated with muscle atrophy may vary over time, and may, especially in combination with other physiological stresses encountered during spaceflight (e.g., hypothermia, medication effects), pose a risk of junctional transmission failure. In the mouse hindlimb unloading model, we were unable to reproduce the full range of ultrastructural changes reported in studies of space-flown animals, and therefore suggest that additional factors (especially reloading injury and/or eccentric contraction injury of atrophied muscle) may have contributed to this disparity. Our evidence to date suggests that transgenic over-expression of IGF-1 in skeletal muscle, which can induce junctional alterations in some systems and is proposed as a potential countermeasure for unloading-induced muscle atrophy, does not exacerbate junctional alterations in this model system. Finally, our preliminary data indicating alteration of intracellular calcium and of calcium-dependent processes within motoneuron terminals suggest the possibility of increasing calcium-binding protein expression as a potential countermeasure for the observed alterations of neuromuscular junctions with hindlimb unloading.

Our comprehensive approach using electrophysiologic and ultrastructural techniques is being extended to determine the junctional effects and tolerability of transgenic overexpression of a calcium-binding protein, parvalbumin, and to determine whether parvalbumin overexpression can

ameliorate motoneuron dysfunction and/or muscle atrophy in mouse models of muscle atrophy and of neuromuscular diseases. Data obtained from this study will be useful in defining the anatomic and physiologic consequences to motoneurons of manipulations which induce muscle atrophy, and will aid in designing further experiments to determine the mechanisms influencing motor unit dysfunction occurring during space travel. Information from this study will be of value to the design and refinement of countermeasures aimed at ameliorating the deleterious effects of microgravity on human motor performance. The results of this work may also provide new insights into important clinical problems such as mechanisms influencing motoneuron dysfunction in devastating degenerative illnesses such as amyotrophic lateral sclerosis, muscle and motor nerve injury encountered in critical care settings, and the design of therapies to retard or prevent muscle atrophy produced by disuse or spinal cord injury.

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I. Research Plan Summary

I. A. Hypotheses, Objectives, and Specific Aims from Original Proposal

The central hypothesis of this study is that **alterations in motoneuron structure and function occur during the process of microgravity-induced muscle atrophy, and that these alterations may significantly influence muscle dysfunction, adaptation, and recovery** from atrophy induced by microgravity. In addition to alterations in moto-neuron activity produced by changes in supraspinal drive and in stretch reflexes during microgravity, we hypothesized that **early structural and functional alterations occur in motoneuron terminals innervating skeletal muscle**. Initiation of these alterations in motoneuron terminals may be influenced by retrograde signals from muscle which induce alterations in intracellular calcium and transmitter release. To begin to test these hypotheses, the following specific aims were proposed:

1. To determine the nature, extent, and time course of motoneuron alterations in a mouse model of microgravity-induced muscle atrophy (hindlimb unweighting), using a variety of electrophysiologic and anatomic techniques.

Rationale: There is considerable anatomical evidence from space-flown animals (Riley et al., 1990; D'Amelio & Daunton, 1992) and from ground-based models of disuse atrophy (e.g., Fahim, 1989) that synaptic vesicle changes, denervation, and junctional remodeling occur in motoneurons innervating atrophying muscle. The mechanism and functional significance of these changes has never been addressed, however, and no systematic study of physiologic parameters or of morphological correlates of synaptic activation has yet been performed in the hindlimb-unloading (HU) model. It is hypothesized that HU will result in an increased incidence of electrophysiologically and/or morphologically altered junctions when compared to controls.

2. To determine whether alterations in motoneuron function in hindlimb-unweighted mice are ameliorated by specific therapeutic interventions which modify muscle atrophy (exercise, growth hormone, IGF-1).

Rationale: Measures which attenuate muscle atrophy induced by unloading may also affect the structure and/or function of the innervating motoneurons. If the signal for motoneuron alteration originates in the muscle, it is expected that such neuronal changes will be ameliorated by measures such as exercise. However, if a GH-induced mechanism such as local IGF-1 production in the muscle is acting as a retrograde signal to induce presynaptic remodelling, it is expected that GH or IGF-1 treatment will result in increased motoneuron remodeling in the presence of diminished muscle atrophy in this model.

3. To determine the effects on motoneuron function of a muscle-derived neurotrophic factor, IGF-1, in hindlimb-unweighted mice with transgenic overexpression of IGF-1.

Rationale: In the setting of exogenous GH supplementation, body weight and total muscle mass increase; in addition, systemic effects of GH cannot be excluded as causes for its effects on muscle atrophy. Furthermore, high-level local production of IGF-1 may be required for full efficacy, both on skeletal muscle and on motoneurons. The ability to increase IGF-1 production specifically in skeletal muscle would represent an important test of this hypothesis. According to the findings of Caroni & Grandes (1990), it is also hypothesized that local overexpression of IGF-1 in skeletal muscle of transgenic mice will lead to increases in motoneuron terminal remodeling.

I. B. Modifications Required upon Selection; Progress and Results

At initiation of this study, two major factors altered the scope and schedule of the proposed work: (a) reduction in overall funding by ~50 percent from the requested amount, and (b) delay in actual availability of funds to the principal investigator until December of the 1st year.

Specific Aim 1. Initially proposed completion: End of year 1.
Modifications to original proposal: Experiments continued through year 3.

(1) Due to lack of funding for a dedicated setup, as well as the time required to train a less experienced postdoctoral associate and technician, the number of muscles studied as well as the number of experimental groups was reduced. Thus, preliminary experiments focused largely on the soleus muscle of HU mice (the muscle most affected in the HU model), with faster muscles (medial gastrocnemius, plantaris) studied as time permits. Initial studies measured effects of unweighting at a single time point of 3 weeks, with additional time points developed as resources permitted. It was expected that these revisions would allow completion of the most important aspects of the original proposal within the allotted time. *An additional limitation is that in order to maximize the data obtained with limited resources, the experiments to correlate physiologic changes in motoneurons with muscle fiber type have been postponed.* A manuscript is in preparation describing the results of this work (**Appendix B**)

(2) Coordination with Dr. Laszlo Siklos (Szeged, Hungary) to assist with E.M. calcium imaging was delayed into year 3 due to a several month down time for repairs of the electron microscope used for these studies. Data analysis continues on this series of experiments, but preliminary results indicate that (a) intraterminal calcium may be increased somewhat with unloading, and (b) the mitochondrial calcium accumulation seen in neurodegenerative disorders such as ALS is not reproduced or mimicked by unloading-induced changes in calcium (see **Appendix A**).

(3) Establishment of normative data and validation of the mouse single-fiber EMG technique has taken additional time and effort during year 2. In order to maintain adequate sample numbers for SFEMG analysis, it has proved necessary to sample muscles on both sides, which has forced the postponement of the evoked release assays planned for the contralateral side. The mouse SFEMG technique has proved extremely useful, however; a manuscript is in review (See **Appendix B**) describing the technique, data from the use of SFEMG are being used to support the manuscript in preparation describing the extent and nature of physiologic alterations of the neuromuscular junction with unloading (see point 1 above).

(4) Characterization of the response of unloaded junctions to muscle stretch was completed, and it is now clear that this property of mammalian junctions appears to be stable even with significant atrophy induced by unloading (**Appendix A**). A manuscript is in preparation describing these negative, but important, findings (**Appendix B**).

(5) It is clear from the ultrastructural studies of junctions in unloaded muscle that unloading in mice, while reproducing some aspects of the changes described in spaceflight, does not reproduce the marked presynaptic ultrastructural abnormalities noted in several studies of space-flown rodents. As we do not believe this disparity to be due simply to differences in technique, we hypothesize that one or more additional factors must be acting in concert with unloading to produce the full range of junctional alterations associated with spaceflight. Most important to address are (1) the time course of junctional changes with unloading, and (2) the possibility that reloading injury or injury from eccentric contraction of atrophied muscle may add to the junctional changes produced by unloading alone (see discussion in **Appendix A**).

Specific Aim 2. Initially proposed schedule: Begin in Year 1; continue through year 3.
Modifications to original proposal:

(1) Due to variable results in collaborating laboratories in demonstrating a consistent effect of exercise and muscle stretch protocols on ameliorating muscle atrophy, we felt that studies of junctional changes originally proposed in this aim were best postponed.

(2) As we now hypothesize that reloading injury or injury from eccentric contraction of atrophied muscle may add to the junctional changes produced by unloading alone (see point 5 of Aim 1 above), we propose to re-approach this aim with the goal of determining whether these protocols may exacerbate junctional changes; data from this work will be critical in refining the design of an exercise protocol that is less susceptible to confounding by such alterations.

(3) In order to take advantage of an ongoing bed rest study at Baylor College of Medicine (A. Leblanc and L. Shackleford, P.I.s), the P.I., in collaboration with Robin Conwit at Johns Hopkins, has begun to gather pilot data on motor unit recruitment in the vastus medialis of human subjects after 17 weeks of bed rest (control, alendronate, and exercise arms). This work is ongoing at the present time.

Specific Aim 3 –
Initially proposed to begin in year 2.
Modifications to original proposal: As in Specific Aims 1-2.

(1) Due to logistic constraints, some of these experiments were initiated ahead of schedule. However, animal availability (particularly with obtaining age-matched controls since many of the parameters measured are known to vary with age) has slowed progress in these experiments. The preliminary data on spontaneous release indicate that transgenic overexpression of IGF-1 in mice does not alter the effect of unloading on motoneuron terminals in atrophied muscle, although it does appear to have a small beneficial effect on the muscle atrophy itself (see **previous reports** and Appendix A). This is an important observation, as we had originaly hypothesized that if IGF-1 was acting as a retrograde signal from unloaded muscle to initiate changes in the motoneuron terminal, it would be possible that successful IGF-1 treatment of muscle atrophy might exacerbate junctional changes and potentially alter neuromuscular transmission.

(2) Due to the increasing evidence for alteration of calcium and calcium-dependent processes at the neuromuscular junction with unloading-induced muscle atrophy, we expanded this aim considerably to focus on the characterization and validation of a transgenic mouse line, developed by Dr. Stanley H. Appel (a co-investigator), that overexpresses the calcium-binding protein parvalbumin in motoneurons. As outlined in **Appendix A**, we have evidence that parvalbumin is overexpressed in motoneurons, that parvalbumin-overexpressing mice have altered calcium homeostasis in motoneurons, and that these mice, when mated with mice that develop a form of amyotrophic lateral sclerosis (motoneuron disease), exhibit significantly delayed disease onset and improved survival. We have as yet identified no safety or tolerability concerns in this model system, and are actively pursuing this approach as a potential countermeasure for motoneuron injury in ALS, and for muscle atrophy and junctional alterations observed with unloading (note proposals in **Appendix B**).

II. Implications of Project Findings for Future Research

Summary. These results, taken together, suggest that **structural and functional changes in the innervating motoneuron may accompany the process of muscle atrophy induced in a mouse model of hindlimb unloading** (HU). It is apparent from the single-fiber EMG studies that such changes are associated with insecure neuromuscular transmission (increased "jitter" suggesting a reduced safety factor). These changes appear to be associated with evidence of altered intracellular calcium in motoneuron terminals. The nature and extent of these changes remains to be better defined by ongoing and planned electrophysiologic and ultrastructural studies, with time course series and interactions with reloading or eccentric contraction-induced injury being high priorities. Preliminary data indicate that IGF-1 overexpression in muscle of transgenic mice is unlikely to worsen the effect of H.U. on at least one aspect of NMJ function. Further studies will be necessary to conclusively rule out a deleterious effect of IGF-1, or to determine whether it has a beneficial effect in this model system. The characterization of mice overexpressing a calcium-binding protein, parvalbumin, in motoneurons and skeletal muscle, has made it possible to begin to pursue the goal of modifying calcium-binding protein expression as a potential countermeasure for motoneuron injury in motoneuron diseases such as ALS, and for muscle and/or neuromuscular junction alterations occurring with unloading-induced atrophy.

Implications for Further Research. Whether the neuromuscular junction alterations described in this model have adaptive or detrimental significance (or possibly both) is not yet fully clear. Increases in transmitter release could help to compensate for deficits in motor unit function (as suggested for myasthenia or myasthenia-like syndromes; Plomp et al., 1995; Sandrock et al., 1997), or to provide trophic influences on muscle function. However, if up-regulation of spontaneous release predisposes to synaptic depression at higher frequencies of stimulation, these same modifications could be detrimental to motor unit performance. Furthermore, if up-regulation of release from motoneurons innervating atrophied muscle is compensatory, it is possible that the mechanisms subserving this change could be highly vulnerable to inadvertent blockade (and consequent decrement in motor unit function) by certain medications. A precedent for this possibility has been hypothesized to account for the unusual sensitivity of patients with myasthenia gravis and other neuromuscular junction disorders to frequently employed medications such as β-blockers, calcium channel antagonists, glucocorticoids (at either pharmacologic doses, or even at physiologic concentrations encountered under stress situations), and aminoglycoside antibiotics. Future studies will need to address these possibilities.

Multiple mechanisms may mediate the unloading-induced NMJ alterations observed in this study. The presynaptic changes are hypothesized to result from a muscle activity-dependent, retrogradely acting factor such as IGF-1 or related signaling molecules (e.g., GDNF). Continuing experiments will begin to test these possibilities. The increases in spontaneous release observed in the plantaris could result from increased intracellular calcium, even in the absence of a clear morphologic alteration in vesicles or evidence of synaptic remodeling. These mechanisms are potential targets for therapeutic intervention.

Altered neuromuscular junction structure and function could result in changes in neurally modulated aspects of muscle physiology, such as acetylcholine receptor (AChR) expression. Limb immobilization in rats has been shown to up-regulate AChR expression in skeletal muscle (Yanez et al., 1996), which could predispose to life-threatening hyperkalemia or to reduced responsiveness to neuromuscular blockers in the setting of a medical emergency requiring intubation. To date, no published reviews of the challenges of inflight surgical interventions have addressed this possibility. Muscle specimens have been collected from ongoing experiments with unloaded mice to begin to address this question, using ^{125}I-α-bungarotoxin binding of AChRs.

Impact on Critical Path Risks.

The work performed has contributed toward understanding neuromuscular junction changes associated with spaceflight. Based on the results of this work, it is clear that neuromuscular transmission, while unlikely to fail in healthy, unloaded muscle, is insecure, and may prove susceptible to failure in the presence of other stresses likely to be encountered in spaceflight (e.g., hypothermia, reloading injury, and commonly administered medications). This would affect muscle performance in the areas of fatigue and loss of muscle power, which would lead to inability to perform specific tasks, and contribute to risk of injury. More research is needed to clarify these risks, and to determine which risks can be avoided by specific interventions such as exercise or pharmacologic interventions, and which risks can be avoided by minimizing exposure to stresses that could impair junctional transmission.

Extensions of the Proposed Work. It is unclear whether the changes observed at the neuromuscular junction in this study also occur at other synapses (e.g., the Ia-to-α-motoneuron synapse subserving the monosynaptic stretch reflex).

The potential involvement of altered intracellular calcium in this model suggests the possibility of testing countermeasures which could alter calcium handling in motoneurons, such as the vitamin D analogs, which are being tested in the laboratory of Dr, Stanley Appel (Alexianu et al., 1998). A transgenic mouse line overexpressing parvalbumin in neurons, developed in the laboratory of Dr. Appel, is presently being characterized electrophysiologically in my laboratory. It is of great interest that the vitamin D analog EB1089, which has been proposed to retard bone loss induced by microgravity, could also have effects on motoneuron or muscle calcium handling.

It also remains to be tested whether the processes defined in the mouse HU model can be extended to human patients. In this regard, the stimulated single-fiber electromyography (SFEMG) studies which are currently being tested in this model have an extensive record of usefulness in the electrodiagnosis of human neuromuscular diseases, and could be immediately applied to testing of human subjects after prolonged bed rest or spaceflights of varying duration. The data obtained from this work are thus likely to be of immediate value in the design of studies aimed at defining the contribution of motoneuronal influences on muscle to overall motor unit function in humans exposed to spaceflight.

Data from this work has also advanced our understanding of the impact of peripheral (muscle and neuromuscular junction) alterations on overall motor dysfunction in devastating human motoneuron diseases such as amyotrophic lateral sclerosis (ALS). Preliminary data generated in part by this NSBRI-sponsored work suggest the feasibility of altering calcium-binding protein expression as a potential countermeasure in patients suffering from ALS, a major area of interest for our laboratories.

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Appendix A - Project Research Data

Functional and structural alterations at the NMJ with hindlimb unloading

In several experimental series, a 3-week period of hindlimb unloading (HU) by tail suspension was well tolerated and produced robust effects on muscle atrophy, with atrophy in the soleus (SOL)

Table 1 – Ranges of muscle atrophy induced by 3-week hindlimb unloading in mice

Body weight	-12% to -21%
Soleus	-41% to -52%
Plantaris	-12% to -17%
Gastrocnemius (total)	-19% to -25%
EDL	-22% in one series

*Ranges for all series, taken together. Effects in young (8-11 wk) ICR mice slightly but not significantly greater than in older (8-10 mo) FVB mice. Atrophy represents difference in wet weight between control and treated groups, not normalized for body weight or tibial size.

muscle consistently exceeding atrophy of more mixed muscles such as the plantaris and gastrocnemius (**Table 1**). Small differences attributed to strain or age of mice were noted across experimental groups. Effects of HU remained statistically significant if corrected for body weight changes, except for changes in the plantaris. These effects are similar to changes reported previously (Criswell et al., 1998). Longer term suspensions (up to 6 weeks) have not resulted in appreciable increases in the degree of muscle atrophy (data not shown). In muscles acutely dissected from 2-3 month ICR mice and studied with intracellular recording techniques in normal calcium saline, spontaneous transmitter release, as estimated by miniature end-plate potential (MEPP) frequency, did not differ significantly in soleus (SOL) muscles of control and 3-week HU mice (**Figure 1**), despite presence of severe atrophy in this muscle.

In striking contrast to the findings from the SOL, significant increases in MEPP frequency were consistently recorded from the plantaris (PLT) following a 3-week period of HU (**Figure 1**). These changes were noted in multiple strains (FVB, ICR, BALB/c) and ages of mice (ranging from 2 to 10 months of age; data not shown). Values for other parameters (recorded *ex vivo* while maintaining the muscle at its minimal *in situ* length; see below), such as muscle fiber resting membrane potential and MEPP amplitude, did not differ consistently among groups, except for a slight decrease in resting membrane potential in soleus muscle fibers, perhaps resulting from effects of atrophy. These data suggest an effect of HU on motoneuron terminals, a presynaptic locus of action, and are consistent with the hypothesis that inactivity-induced alterations (perhaps retrograde signals from inactive muscle) result in modification of neuromuscular junction (NMJ) function. Further studies using longer-term (~6 week) suspensions indicate that with continued unloading, MEPP frequency changes may return to near control levels in the PLT muscle, while actually increasing slightly (~20%; $p < 0.01$) in the SOL. These data highlight the importance of defining the time course of the observed changes, a key part of the work proposed in **Aim 1**.

To correlate the observed changes in transmitter release with ultrastructural changes at NMJs, we transcardially perfused ICR mice (control vs. HU for 3 weeks) following excision of muscles for *ex vivo* physiologic studies. A striking change in the ratio of postsynaptic to presynaptic membrane surface of randomly selected terminal boutons (see **General Methods**), evident on nearly every pair of micrographs examined, was present in the SOL muscle (**Figure 2**). As motoneuron terminal bouton size did not appear to change in this study, and the number of junctional folds appeared to be clearly reduced, this change was interpreted as a postsynaptic alteration resulting from unloading. As can be seen in **Table 2**, this reduction in the post:pre surface ratio was approximately 35%, comparable to the degree of whole muscle atrophy in the SOL when adjusted for body weight changes with HU. We did not observe significant changes in the PLT with respect to surface ratios, or in either muscle with respect to Schwann cell envelopment of the terminal (a sensitive indicator of denervation in our previous studies [Siklos *et al.*, 1996] as well as in the literature), synaptic vesicle density, density of vesicles around active zones (not shown), or mitochondria in the nerve terminal (**Table 2**). Very few NMJs with junctional folds denuded of overlying terminals or enveloped by Schwann cells, and none with prominent presynaptic alterations, were noted in these assays, in contrast to the findings reported in space-flown animals (e.g., Riley *et al.*, 1990a, Babakova *et al.*, 1992; D'Amelio & Daunton, 1992). As we do not believe that this discrepancy can be explained solely by differences in sampling technique, we hypothesize that differences among the models (e.g., the effects of reloading injury in atrophied muscle) may contribute to the higher incidence of abnormal NMJs observed in space-flown animals. Tests of this hypothesis form the basis for the proposed work in **Aim 2** of this study.

To better assess the overall function of the NMJ in muscles of mice subjected to HU, we developed and validated the technique of stimulated single-fiber electromyography (S-SFEMG) for use in the mouse gastrocnemius (Gooch & Mosier, 2000). S-SFEMG jitter, measured as the mean consecutive difference (MCD) in latency between successive single muscle fiber action potentials evoked by nerve stimulation, correlates well with the safety factor for neuromuscular transmission (e.g., Lin & Cheng, 1998). Following 3 weeks of HU in anesthetized ICR mice, we observed, at low rates of stimulation (2/sec), a striking increase in mean jitter values (by ~200%) in single fibers of unloaded gastrocnemius muscle (**Figure 3**), suggesting insecure neuromuscular transmission. Although actual blocking (failure) of transmission was noted in some fibers in this study, we would caution against directly extrapolating these results to unanesthetized human subjects. However, these results raise the possibility that, especially under

predisposing conditions (e.g., drug effects, thermal injury, etc.), failure of neuromuscular transmission could become clinically significant in atrophied muscle. It is also worth noting that increases in jitter have been reported in cast-immobilized soleus muscles in human subjects (Grana *et al.*, 1996), highlighting the potential importance of the present findings.

As previously noted, what is not known is the time course of unloading-induced alterations in NMJs, which could vary among muscles due to differences in a number of factors, including fiber type distribution, mechanical stretch, and patterns of activation in the unloaded state. Furthermore, a precise correspondence between functional and ultrastructural changes with unloading remains to be established (*Reviewers please note*: an apparent mismatch between functional changes and structural alterations at NMJs has been reported by others [see Colman *et al.*, 1997; Prakash *et al.*, 1999], which may reflect the order of causation, or differences between underlying mechanisms). The time course measurements and correlative studies proposed in **Aims 1 and 2** are designed to quantitatively address these questions, as well as better defining mechanisms underlying the striking changes in NMJ function associated with muscle unloading.

Muscle-motoneuron interactions at the neuromuscular junction

We examined the effects of muscle stretch on spontaneous transmitter release in hindlimb muscles from unloaded mice, both to rule out a potential confounding variable for the MEPP frequency studies above, and to investigate the possibility of a potential mechanical or contact-mediated muscle-motoneuron interaction which could alter NMJ physiology with unloading. In general, the mammalian skeletal NMJ is insensitive to muscle stretch (see **Background** above). Our findings in SOL and PLT, based on *ex vivo* stretch from the minimal muscle length measured *in situ* (comparable to the *ex vivo* relaxed length) to the maximal length measured *in situ*, are consistent with these reports, with no significant effects of muscle stretch on MEPP frequency observed in either muscle (data not shown). However, in the EDL muscle, which by our measurements is always held in a slight amount of tension (minimal *in situ* length exceeding fully relaxed length by ~5%), we noted a *decrease* of MEPP frequency with unloading, and a further reduction of MEPP frequency with muscle stretch within the physiologic range (**Figure 4**), with no apparent effect on resting membrane potential or MEPP amplitude among any of the tested groups. These data suggest that muscle stretch effects, at least acutely, do not account for the HU-associated changes in spontaneous release in the posterior compartment muscles (SOL and PLT) tested above. However, the data raise the possibility that a small degree of stretch sensitivity of release may become apparent in a mixed muscle of the anterior compartment (EDL), and studies of NMJ function with HU in this muscle must take into account the potential effects of mechanical influences. Whether these effects of muscle stretch are associated with changes in evoked release, or other alterations in the NMJ, is not known; nor are the NMJ effects of chronic, passive muscle stretch *in vivo* understood at the present time. [*Reviewers please note*: the small effects of muscle stretch on spontaneous release in these experiments should not be confused with the dramatic effects of muscle stretch on evoked release at amphibian NMJs, where enhancement of release is of sufficient magnitude to act as a peripheral amplifier of the stretch reflex (e.g., Chen & Grinnell, 1995). We believe that our data confirm previous observations of the general insensitivity of mammalian NMJs to stretch effects, but are of value in that they suggest caution in the interpretation of small differences in observed NMJ function where muscle length in the assay system is not controlled].

Changes in intracellular Ca^{2+} with hindlimb unloading

In our ultrastructural studies of NMJs to date, we have not observed changes in the number of active zones, vesicle density around active zones, or evidence of sprouting to account for the observed increases in MEPP frequency in the PLT muscle with HU. An attractive candidate mechanism for increased spontaneous release in this model system is an increase in intracellular calcium concentration at the motoneuron terminal. A large body of published reports implicate intraterminal calcium as a key regulating signal for neurite extension, growth cone guidance and interaction with target muscle fibers, effects of muscle-derived trophic factors, and sprouting (for recent discussions see Boulanger & Poo, 1999; Graf *et al.*, 1999; Lautermilch & Spitzer, 2000; Santafe *et al.*, 2000). As an initial test of the hypothesis that increased intraterminal calcium contributes to the NMJ changes observed with HU, we employed electron microscopy to assay intracellular calcium using an oxalate-pyriantimonate precipitation technique (e.g., Siklos *et al.*, 1996, 1998). Preliminary data from this approach (generated from 4 of an anticipated 10 pairs of mice) suggest that HU of 3 weeks' duration may be associated with a 44% increase in cytosolic Ca^{o} in motoneuron terminals of the PLT muscle, while producing little or no changes in terminals of the SOL muscle (-8%). These differences, while not statistically significant in this interim analysis of < 1/2 of the planned experiments, appear to parallel the changes in MEPP frequency observed with HU in the PLT and SOL muscles (**Figure 1**). Further work is needed to confirm these interim findings, and to better define the magnitude of the observed effects, and will be addressed in **Aim 1**.

These data, taken together with the extensive evidence implicating intracellular calcium as a key signal for neuromuscular junction formation and modification, suggest that intracellular calcium may be elevated in motoneuron terminals of muscles subjected to hindlimb unloading. Elevations in intracellular calcium, while not observed in studies to date to reach the levels believed to be associated with mitochondrial toxicity in disorders such as amyotrophic lateral sclerosis (Siklos *et al.*, 1996), may be sufficient in NMJs of hindlimb-unloaded animals to act as physiological signals for junctional remodelling, and to induce significant alterations in presynaptic terminal function. The time course of these changes with unloading or reloading is presently unknown, and forms part of the basis for investigations proposed in **Aims 1 and 2** of this study. It is also not known whether interventions known to stabilize neuronal Ca^{2+} levels *in vivo*, such as increased expression of the Ca^{2+}-binding protein parvalbumin, can attenuate the NMJ alterations observed with muscle unloading; testing of this hypothesis forms the basis for the experiments proposed in **Aim 3** (see below).

Calcium-binding protein overexpression, Ca^{2+} homeostasis, and neuroprotection in motoneurons

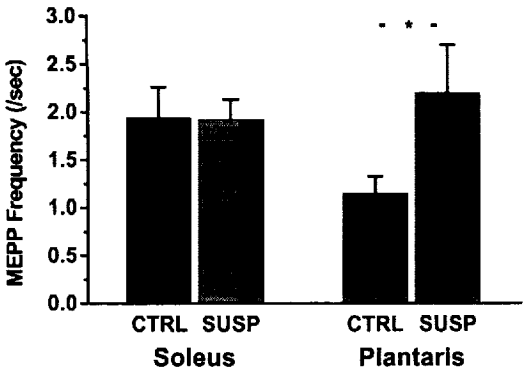
To test the hypothesis that overexpression of Ca^{2+}-binding proteins can modulate neuronal calcium perturbations and influence calcium-dependent processes in mice, we generated transgenic mice expressing rat parvalbumin (PV) under control of the rat calmodulin II (CaMII) promoter (Beers DR, *et al.*, in preparation). Briefly, four founder mice were identified by Southern blots; of these, two (lines 12 and 14) were bred to homozygosity. RT-PCR detected high levels of rat PV transcripts in spinal cord, skeletal muscle, and liver; *in situ* hybridization with an antisense probe confirmed PV mRNA expression in lumbar, cervical, and hypoglossal motoneurons of PV transgenic lines, but not in control mice (**Figure 5**). PV mRNA expression was not observed in glial cells adjacent to motoneurons. Western blots confirmed PV protein overexpression in spinal cords of PV transgenic mice (**Figure 6**), to a level of ~5x controls. Quantitative determination of PV protein expression in skeletal muscle of PV transgenic mice is currently in progress. Immunohistochemistry confirmed significant PV protein expression in spinal cord and hypoglossal motoneurons of both transgenic lines, but little or no expression was detected in motoneurons of control mice (data not shown). PV overexpression did not appear to alter the expression of another calcium-binding protein, calbindin-D_{28K}, in motoneurons (D.R. Beers, pers. comm.).

Both PV-overexpressing lines are similar in weight and gross morphology to the B6/SJL background strain. In a small number of animals, we have observed no obvious changes in S-SFEMG jitter with parvalbumin overexpression (mean ± SD: 12 ± 4 μs for PV line 14; 10 ± 3 μs for wild type controls, $p = 0.17$), suggesting that the safety factor for overall neuromuscular transmission is not greatly altered with parvalbumin overexpression. We have not observed increases in susceptibility to hypoventilation with usual doses of anesthesia in the parvalbumin transgenic mice, again suggesting that no gross dysfunction of motor units occurs with parvalbumin overexpression. A trend toward enhanced Schwann cell envelopment of boutons was noted in PV transgenic mice with respect to controls (borderline significance, $p = 0.05$), while clear increases in the postsynaptic: presynaptic surface ratio were evident in PV transgenic mice ($p < 0.001$), suggesting the possibility that PV overexpression may influence NMJ remodelling. Denuded postsynaptic folds or altered vesicle density in terminals was not observed to an appreciable extent in control or PV transgenic mice. Although not quantified at present, the parvalbumin transgenic lines appear to exhibit similar levels of motor activity to controls, and do not exhibit spontaneous seizure activity on EEG screening (Noebels JL, pers. comm.). Thus, there is no evidence at present that gross locomotor activity or neuromuscular function are adversely affected by long-term transgenic overexpression of parvalbumin.

To determine whether PV overexpression was associated with enhanced calcium homeostasis in motoneurons, we measured intracellular calcium (by oxalate-pyriantimonate precipitation) and spontaneous transmitter release (by intracellular recording) following in vivo passive transfer of sera from patients with ALS. This intervention, which causes elevations in intraterminal calcium and MEPP frequency at NMJs within 24 hours *in vivo* (Engelhardt *et al.*, 1995; Mosier *et al.*, 2000), produced robust effects in wild-type control mice, but failed to alter intracellular calcium or transmitter release in either line of PV transgenic mice (not shown). *In vitro* alteration of bath calcium over a log-unit range increased Ca^{2+}-dependent spontaneous release to a much lesser extent in nerve-muscle preparations from PV transgenic mice than from controls (**Figure 7**). These data, taken together, suggest considerable attenuation of intracellular calcium increases and of changes in spontaneous release, a calcium-dependent process, in motoneuron terminals of parvalbumin-overexpressing mice.

To test the hypothesis that parvalbumin overexpression in motoneurons confers protection against motoneuron injury, parvalbumin transgenic mice were crossbred with transgenic mice overexpressing the G93A mutation of human superoxide dismutase (SOD1), a mouse model of familial amyotrophic lateral sclerosis (ALS). In doubly transgenic mice overexpressing mutant SOD1 (G93A) and parvalbumin, highly significant increases in overall survival were observed (**Table 3**), principally accounted for by a delay in the age of onset of clinical evidence of motoneuron disease. Preliminary data (D.R. Beers and S.H. Appel) suggest a trend toward improved motoneuron cell body survival in these doubly transgenic mice, compared to mutant SOD1 transgenic controls. These effects have persisted over ~8 generations, and are present in both transgenic lines, suggesting that differences in genetic background or site of transgene insertion are unlikely to be confounding factors. These encouraging results suggest that transgenic overexpression of parvalbumin is associated with clinical protection in a model of motoneuron injury, and may also be neuroprotective in these mutant SOD1 mice. Whether overexpression of parvalbumin, either in motoneurons or in skeletal muscle, can protect against muscle atrophy or NMJ alterations associated with unloading or reloading, is not known, and testing this hypothesis forms the basis for the proposed work in **Aim 3**.

Figure 1. Spontaneous Release at Neuromuscular Junctions of Control and Tail-Suspended ICR Mice

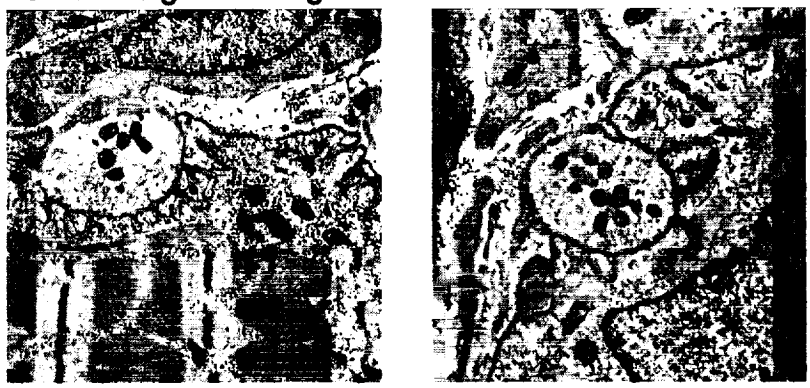


Mean + 95% CI of fibers tested; * p < 0.0005

Figure 1. Summary of intracellular recordings of MEPP frequency from randomly selected fibers of tested muscles. Data from ~6 experiments per group. Differences between grand means for the PLT are also significant ($p < 0.01$).

Figure 2. SOLEUS

CTRL Weight-Bearing **SUSP - UNLOADED**



Representative boutons at neuromuscular junctions after 3 weeks of hindlimb unloading in ICR mice. x20,000.

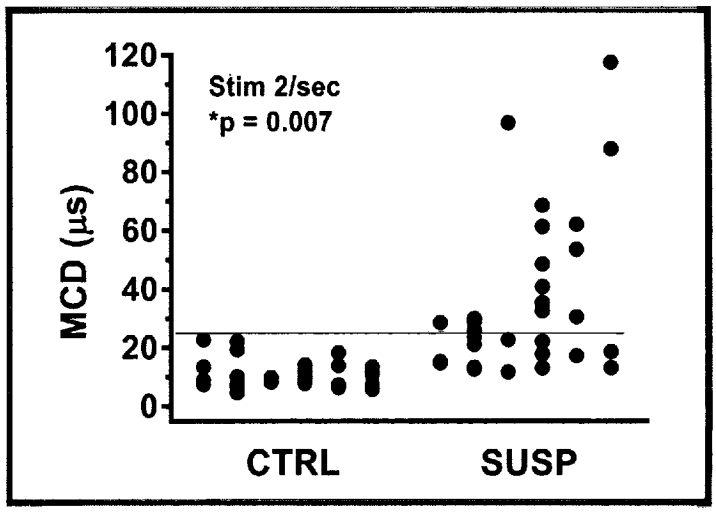
Table 2. Ultrastructural Parameters at Neuromuscular Junctions of ICR Mice After 3 Weeks Hindlimb Suspension

	SOLEUS Control	Suspended	PLANTARIS Control	Suspended
Surface ratio (post : pre)	4.0 ± 1.9	2.7 ± 0.3 *	4.3 ± 1.0	5.2 ± 1.0
Schwann cell envelopment of terminals (% of pre-synaptic bouton surface)	37.3 ± 7.1	40.3 ± 5.9	34.1 ± 2.6	41.4 ± 11.0
Synaptic vesicle density (arbitrary units) **	1.6 ± 0.4	1.6 ± 0.1	1.6 ± 0.4	1.6 ± 0.5
Mitochondrial volume fraction (% terminal vol.)	15.2 ± 3.6	15.7 ± 3.3	18.2 ± 4.7	14.5 ± 1.9

Data are mean ± SD of 5 muscles per group. *Significantly different from control, p = 0.012, 2-tailed *t* test.
**Identical scaling used for all terminals. This measure should be regarded as semiquantitative since the average diameter of vesicles is comparable to the section thickness, and thus an component of variability in apparent vesicle density due to minor, normally encountered variations in section thickness could be introduced.

FIGURE 3. HINDLIMB UNLOADING INCREASES S-SFEMG JITTER IN GASTROCNEMIUS MUSCLES OF ICR MICE

Data points represent jitter of single fibers of gastrocnemius muscles in 10 wk ICR mice after 3 weeks of hindlimb unloading. Each column depicts data from all fibers tested in an individual experiment. A total of 32 fibers from controls and 30 fibers from tail-suspended mice were tested. Horizontal line is drawn at mean + 3SD of control data.



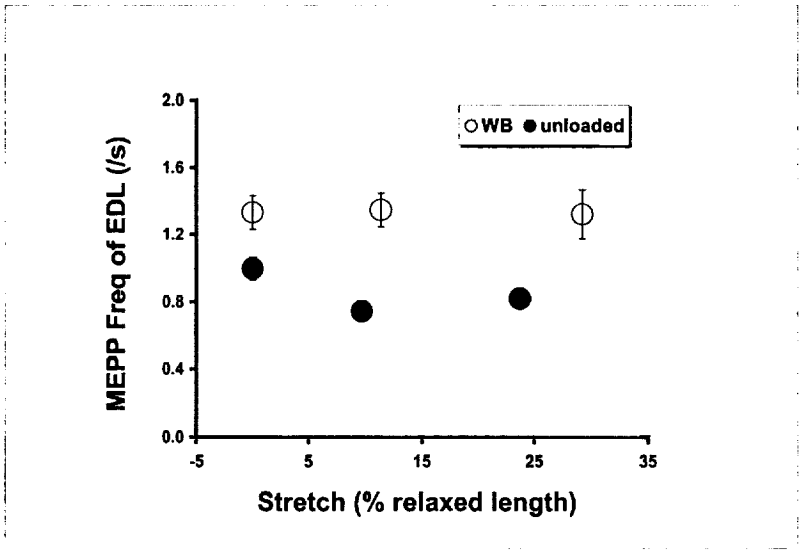


Figure 4. Data from intracellular recordings of MEPPs in randomly selected fibers from EDL muscles of 8 weight-bearing (WB) controls and 8 mice subjected to HU for 4 weeks. Each muscle is tested at 3 different lengths; data points represent mean ± SE of experimental means, with 5 fibers sampled at each length tested. Differences between WB and HU groups are significant ($p < 0.001$); differences with increasing stretch (compared to data from fully relaxed EDL in the HU group) are also significant ($p < 0.01$).

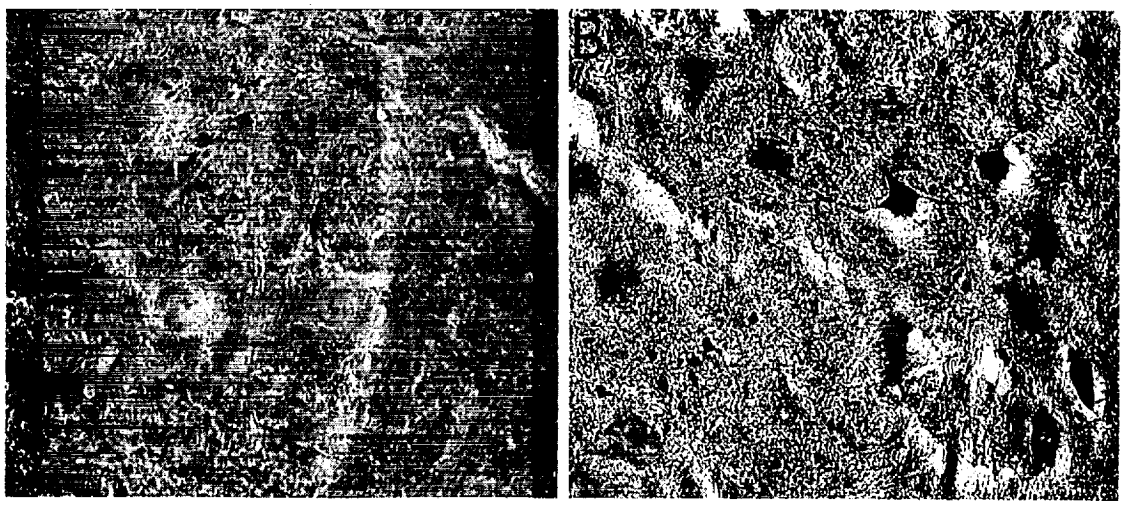


Figure 5. Localization of rat parvalbumin mRNA in the spinal cords of control and Tg-mice by *in situ* hybridization. No *in situ* hybridization signal for parvalbumin mRNA was observed in lumbar spinal cord neurons of control mice using a anti-sense parvalbumin probe (A). Using the antisense parvalbumin probe, *in situ* hybridization signal for parvalbumin mRNA was observed in lumbar spinal cord neurons of parvalbumin transgenic (line 14) mice (B).

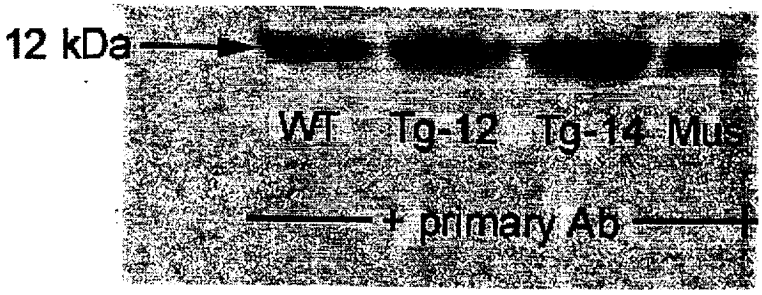


Figure 6. Western analyses of parvalbumin protein expression in mice. Immunodetection of parvalbumin protein in spinal cord extracts of wild type (lane 1) and parvalbumin transgenic mice (lanes 2 and 3). Parvalbumin protein expression is increased in both parvalbumin transgenic mouse lines. Lane 4 contains protein extracted from muscle as a positive control for parvalbumin. Omission of primary antibody led to no detectable signal (not shown). 20μg partially purified protein was loaded into each lane according to the Laemmli method.

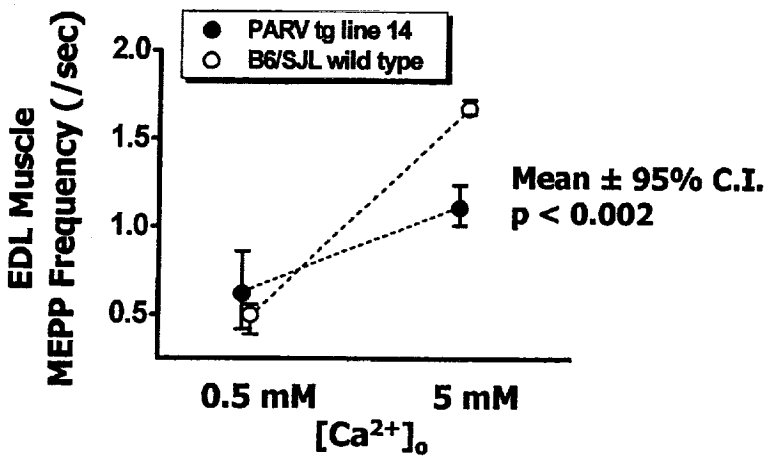


Figure 7. Data from intracellular recordings of randomly penetrated muscle fibers in EDL. Each data point represents mean ± 95% CI of experimental means from 4 animals tested.

Table 3. Comparison of Disease Onset and Survival Between SOD1/Parv and SOD1 Transgenic Mice

	Human Mutant SOD1 (G93A) X Parv Tg Mice (n=18)	**Human Mutant SOD1 (G93A) Transgenic Mice (n=15)**	***p* value – Student's *t* test (two tailed)**
Disease Onset (days)	112 ± 5	96 ± 4	0.02
Survival (days)	146 ± 5	132 ± 2	0.01
Disease Duration (days)	34 ± 7	35 ± 5	0.98

Onset of disease was determined when there was a 2 standard deviation drop (on a neuromuscular rating scale adminstered by 2 blinded investigators) below the previous 3 weekly means. Survival endpoint was defined by occurrence of death or sufficient weakness to prevent ambulation or oral nutrition. Animals were identified by PCR using specific parvalbumin or mutant SOD1 primers.

Appendix B. PUBLICATIONS ARISING FROM THIS WORK (Partly Supported by NSBRI)

Manuscripts.

Mosier, D.R., Siklós, L. & Appel, S.H. Resistance of extraocular motoneuron terminals to effects of amyotrophic lateral sclerosis sera. *Neurology* **54**, 252-255 (2000).

Gooch, C.L. & Mosier, D.R. Stimulated single-fiber EMG in the mouse: techniques and normative data. *Muscle & Nerve*, in review.

Mohamed, A.H., Mosier, D.R., Zou, L., Siklós, L., Alexianu, M.E., Engelhardt, J.I. & Appel, S.H. Identification of high affinity FcγRI receptor in motor neurons: roles in immunoglobulin uptake, immunoglobulin-mediated calcium increase and neurotransmitter release. (*Submitted*)

Beers, D.R., Ho, B.-K., Siklós, L., Alexianu, M.E., Mosier, D.R., Mohamed, A.H., Otsuka, Y., Kozovska, M.E., Smith, R.G. & Appel, S.H. Parvalbumin maintains calcium homeostasis and delays disease in ALS mice. (*Submitted*)

Pang, J. & Mosier, D.R. Muscle stretch and transmitter release at mouse neuromuscular junctions following hindlimb unloading. (*In preparation*)

Mosier, D.R., Siklós, L., Gooch, C.L., Pang, J., Gordon, S. & Booth, F.W. Alterations in neuromuscular junctions associated with muscle atrophy induced by hindlimb unloading. (*In preparation*)

Abstracts and Presentations.

Effect of hindlimb unloading on spontaneous transmitter release from motoneuron terminals. D.R. Mosier, L. Siklós, S. Gordon & F. Booth. National Space Biomedical Research Institute meetings (Baylor College of Medicine, 16 March 1998; Lake Conroe, TX, 9 June 1998).

Effect of hindlimb unloading on spontaneous transmitter release from motoneuron terminals. D.R. Mosier, L. Siklós, S. Gordon & F. Booth. *Ann. Neurol.* **44**, 458 (1998).

Alterations in neuromuscular junctions associated with muscle atrophy induced by hindlimb unloading. D.R. Mosier, L. Siklós, C.L. Gooch, S. Gordon & F.W. Booth. USRA/NASA 1st Biomedical Investigators' Meeting (January 11-13, 1999).

Effects of amyotrophic lateral sclerosis sera on transmitter release and intracellular calcium at neuromuscular junctions of mice deficient in the immunoglobulin Fc receptor. D.R. Mosier, L. Siklós, J.I. Engelhardt, A.H. Mohamed & S.H. Appel. *Neurology* **52** (Suppl. 2), A533 (1999).

"SFEMG in the mouse: techniques and normative data." Gooch CL, Mosier D.R. *Muscle Nerve* **22**, 1325 (1999).

Quantitated myotonic activity supporting a transgenic mouse model of myotonic dystrophy. C.L. Gooch, D.R. Mosier, T. Ashizawa, J. Noebels, M.I. Coolbaugh, G. Brook, T. Fortune, C.T. Caskey, M. Meistrich, M.J. Siciliano & D.G. Monckton. *Muscle Nerve* **22**, 1324-1325 (1999).

Transgenic mouse expression of parvalbumin in motor neurons alters calcium homeostasis and prolongs SOD G93A transgenic mouse survival. S.H. Appel, M. Alexianu, D. Beers, R.G. Smith, D. Mosier, M. Kozovska & L. Siklós. *Ann. Neurol.* **46**, 463 (1999).

Enhanced parvalbumin expression attenuates motoneuron responses to calcium elevation. D.R. Mosier, J. Pang, L. Siklós, D.R. Beers, C.L. Gooch & S.H. Appel. *Neurology*, **54** (Suppl. 3), A305 (2000).

"Basic neurophysiology of the presynaptic terminal: modifiability in normal and disease states." D.R. Mosier. Single Fiber EMG Special Interest Group, American Academy of Electrodiagnostic Medicine 47th annual meeting (Philadelphia, Sept. 16, 2000). [Invited lecture]

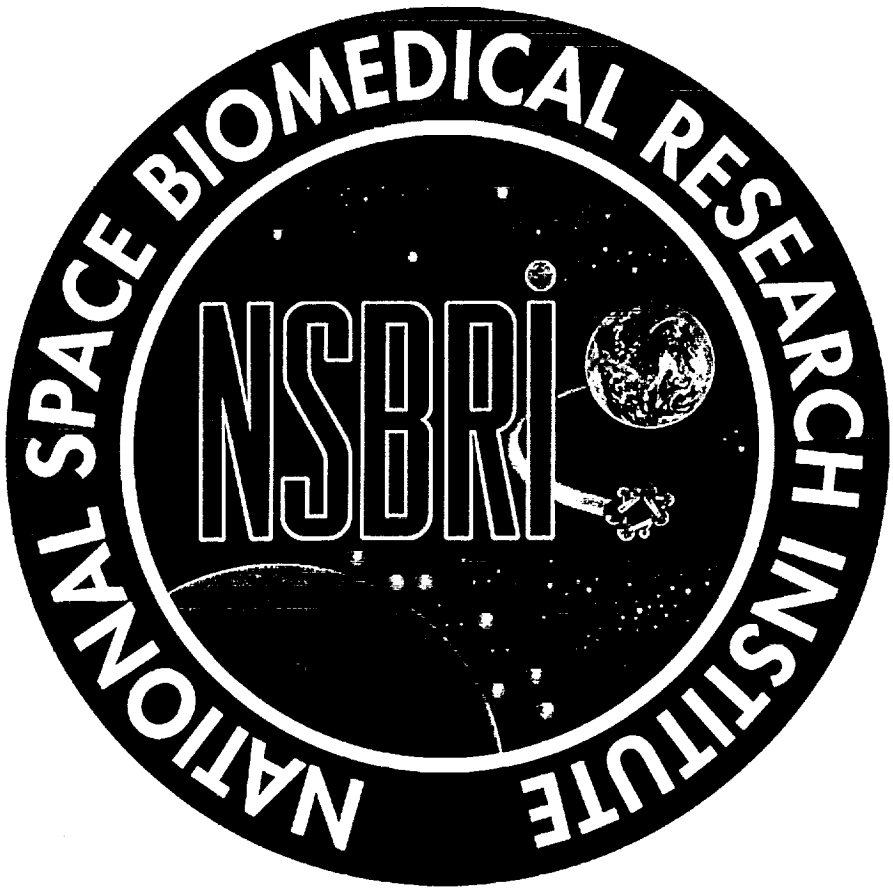
Grant Submissions

1.
NIA/NINDS
"Drug inhibition of beta-amyloid induced immune responses"
Dana Giulian, principal investigator
Dennis R. Mosier, co-investigator
Matt Nance, co-investigator
Paul Schulz, co-investigator
(4-01 to 3-04) Submitted 6/00, funding decision in 1/01
Submission based in part on preliminary data acquired during NSBRI-supported studies.

2.
NINDS RFA for ALS
"The role of calcium in ALS motoneuron vulnerability"
Stanley H. Appel, principal investigator
Dennis R. Mosier, co-investigator
R. Glenn Smith, co-investigator
Laszlo Siklos, collaborator
David R. Beers, co-investigator
Luis V. Colom, co-investigator
(4/01 to 3/06) Submitted 7/00
Submission based in part on preliminary data acquired during NSBRI-supported studies.

3.
VA Merit Review proposal
"Altered calcium regulation in motoneuron disease"
Dennis R. Mosier, principal investigator
Laszlo Siklos, co-investigator
S.H. Appel, co-investigator
David R. Beers, co-investigator
10/01 to 9/04 In local VA review for submission 12/00
Submission based in part on preliminary data acquired during NSBRI-supported studies.

Appendix C
Final Program & Project Reports
for the
Initial NSBRI Research Program
October 1, 1999 – September 30, 2000

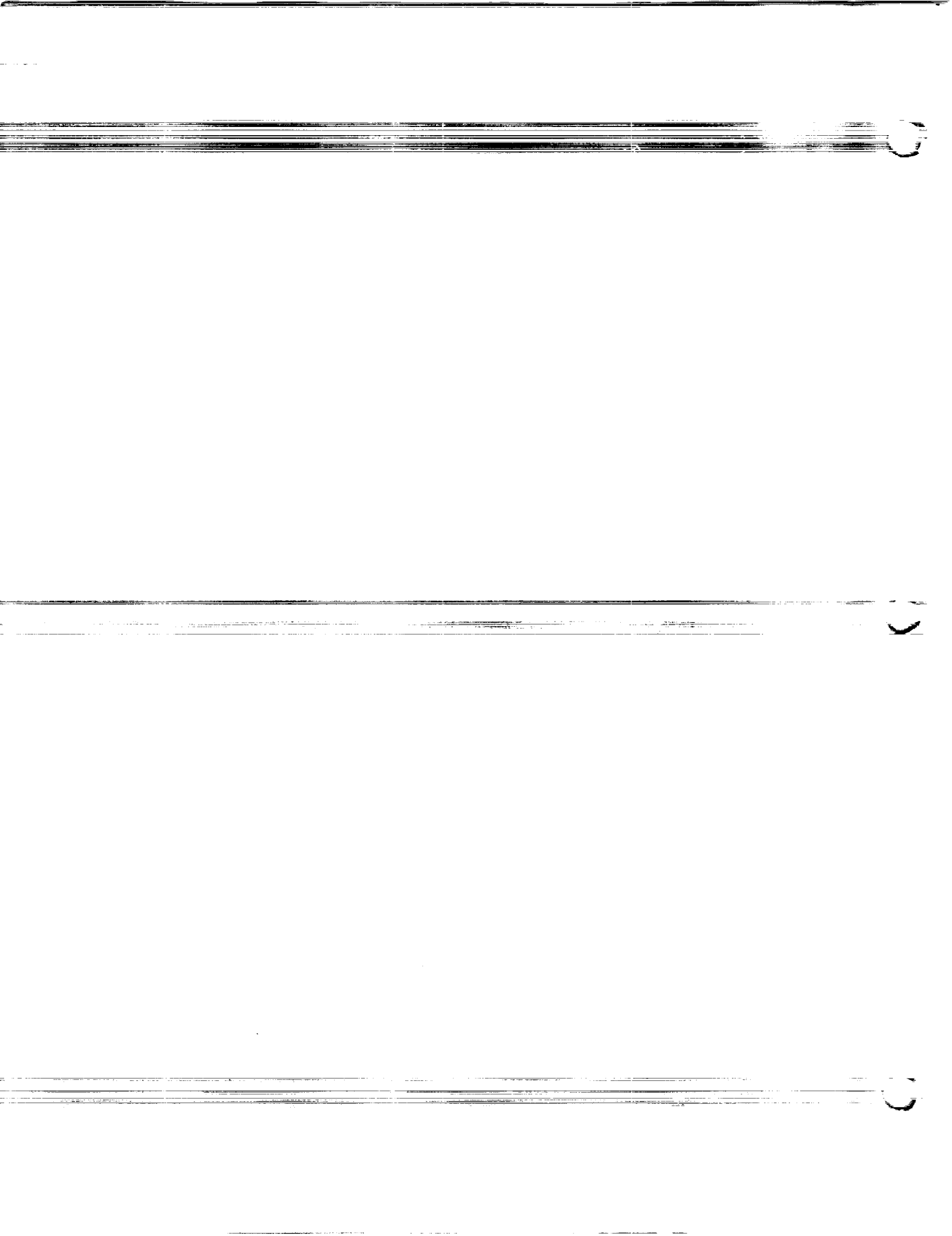


Volume Two:
Neurovestibular Adaptation
Radiation Effects
Technology Development
Synergy Projects

NSBRI RESEARCH PROGRAM
NEUROVESTIBULAR ADAPTATION

Team Leader: Oman, C. M. MIT

Shelhamer, M. J.	**PI**	**Hopkins/SOM**	**Context-Specific Adaptation Of Gravity-Dependent Vestibular Reflex Responses**
Goldberg, J.	CO-I	Baylor	
Minor, L. B.	CO-I	Hopkins/SOM	
Paloski, W. H.	CO-I	NASA JSC	
Young, L. R.	CO-I	MIT	
Zee, D. S.	CO-I	Hopkins/SOM	
Oman, C. M.	**PI**	**MIT**	**Visual Orientation in Unfamiliar Gravito-Inertial Environments**
Beall, A. C.	CO-I	UC, Santa Barbara	
Howard, I. P.	CO-I	York University	
Shebilske, W. L.	CO-I	Wright State University	
Taube, J. S.	CO-I	Dartmouth College	
Wall, C. C.	**PI**	**Harvard**	**Advanced Techniques for Assessment of Postural and Locomotor Ataxia, Spatial Orientation and Gaze Stability**
Bloomberg, J. J.	CO-I	NASA JSC	
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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

FINAL PROGRAM REPORT

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PROGRAM EXECUTIVE SUMMARY

NSBRI's neurovestibular adaptation research program supports research aimed at developing scientifically-based countermeasures against the vestibular problems associated with long duration space flight: space motion sickness, disorientation, oculomotor deficits, postflight postural instability and gait ataxia. Neurovestibular problems typically arise first when astronauts transition from 1-G to 0-G, unfortunately just when their physical and cognitive performance is critical for mission success and safety. Similar problems are expected on exploration class missions when astronauts make the transition from 0-G to partial G, or from 0-G to an artificial gravity environment. Our research also has potentially important terrestrial applications: An estimated two million American adults summer from chronic impairment of dizziness or difficulty with balance, and one quarter of emergency room visits include a complaint of dizziness. Balance-related falls account for more than one half of accidental deaths in the elderly.

The NSBRI neurovestibular adaptation research program addresses five major space neurovestibular risk areas, as identified by NASA Life Science's Critical Path Risk analysis project:

1. Disorientation and reduced performance on cognitive and physical tasks, including vehicle egress, especially during/after g-level changes (associated with acute spontaneous and head movement contingent vertigo, nystagmus, oscillopsia, saccadic errors, reduced dynamic visual acuity, spatial memory problems).

2. Impaired neuromuscular coordination and/or strength (gait ataxia, postural instability).

3. Impaired cognitive and/or physical performance due to spatial disorientation, motion sickness symptoms or treatments (including short term memory loss, reaction time changes, drowsiness, fatigue, torpor, irritability, ketosis) as a result of changes in g-level, or use of artificial gravity.

4. Autonomic dysfunction (including cardiovascular, respiratory, gastrointestinal, sleep and mood changes) which may be of vestibular origin.

5. Permanent impairment of orientation or balance function due to microgravity or radiation (causing chronic imbalance, gait ataxia, vertigo, chronic vestibular insufficiency, poor dynamic visual acuity)

The goals of the program are to develop countermeasures that ultimately will to allow crewmembers to:

- Avoid disorientation
- Meet physical requirements of emergencies
- Treat motion sickness without side effects
- Safely control vehicles and systems.

Nine interrelated, countermeasures-oriented research themes currently define the scope of the neurovestibular program:

1. Adaptive Generalization and Context-Specific Adaptation
2. Artificial Gravity
3. Visual (multisensory) orientation, navigation and spatial memory
4. Drug countermeasures
5. Postflight locomotion and gaze assessment
6. Neurovestibular rehabilitation
7. Vestibular effects on autonomic function

8. Effects of weightlessness, stress, isolation, immobilization, radiation and diet on vestibular function.
9. Potential mechanisms for and diagnosis of irreversible neurovestibular changes.

During the 1981-1997 Shuttle era, space neurovestibular research focused on understanding the effects of unweighting of the otoliths on the vestibulo-ocular reflex (VOR), predicting space sickness susceptibility, and measuring postflight postural stability while standing. NSBRI's current early ISS-era neurovestibular research program is investigating context specific pre-adaptation, preflight visual orientation and 3D spatial memory training countermeasures, and improving our ability to assess postflight locomotion and gaze control problems. Research is being conducted at the cognitive, behavioral, system, organ, and cellular level, using quantitative techniques in both humans and animals. The work is interdisciplinary, and involves collaborations between investigators at multiple institutions.

Our goal is to achieve risk reduction by developing scientifically based countermeasures. Basic research projects must plausibly lead in that direction. Once specific countermeasures are proposed, they will have to be proven safe, effective and practical, and their potential impact on other physiological systems understood. NASA and NSBRI define the countermeasures development process in terms of three phases, and nine levels of readiness, which range from basic research (levels 1-3) through countermeasure feasibility and development (levels 4-6) to ground evaluation and flight validation (levels 7-9). Most NSBRI research teams' activity falls in the 1-7 range.

During the Institute's first three years, neurovestibular adaptation research was conducted by a team of 21 investigators from 11 institutions, organized into three projects. An introduction to these projects is available at www.nsbri.org and also from the Neurovestibular Team web site mvl.mit.edu/Neurovestibular/Pages/home.html. Final project reports are available from NSBRI which detail the scientific accomplishments of the three projects and their implications for future research are summarized and detailed.

This report describes the development of NSBRI's neurovestibular program research strategy and the "critical path" risks which motivate it. It also provides a programmatic level review of each project's research questions, an overview of what was achieved, and how far each projects moved the neurovestibular discipline forward in terms of countermeasures readiness.

Context-Specific Adaptation of Gravity-Dependent Vestibular Reflex Responses. (M.J. Shelhamer, Johns Hopkins University, and 5 co-investigators.)
Can vestibular reflexes be pre-adapted (pre-conditioned) in context specific ways, so astronauts can rapidly transition between Earth, weightlessness, rotating spacecraft (artificial gravity) and planetary gravity, with minimal disorientation and performance impairment ? This basic research project succeeded in obtaining fundamental evidence on several types of context-specific adaptation in both human and animal subjects. It was demonstrated that g-level and eye position can be used as a context cue for changing the size of saccadic and smooth pursuit eye movements, and that saccadic adaptation can persist for many months. G-direction can provide a context cue for adapting the Linear Vestibulo Ocular Reflex (LVOR). In both cases, the evidence suggests that the more relevant the context cue is to the response being adapted, the more effective it is. Adaptation of the Vestibular Coriolis oculomotor response in a rotating environment – and retention of it - is of particular importance to our artificial gravity research theme. It was shown that three ten-minute periods of out-of-rotation-plane head movements produced measurable reduction of inappropriate eye movements, and adaptation was retained one week later. Some support for context specificity was found: subjects did not experience motion illusions when head movements were made in a non rotating environment. In other experiments, it was shown that the human vestibulo-collic reflex adapts much more quickly to artificial increases in head inertia after several practice sessions on consecutive days. Some progress was made using primate models to understand the underlying physiology of adaptation: LVOR adaptation was found to be specific to the frequency used. There was no correlated change in static ocular torsion resulting from head tilt. Floculectomy permanently reduced the LVOR, even after the angular VOR had recovered. Human cerebellar patients show comparable deficits. The investigators note that the "0-g flashbacks" anecdotally reported by some cremembers are consistent with the notion of dual-

state adaptation. They have also pointed out that cerebellar adaptation failure might account for a number of the oculomotor problems reported in the Russian literature after long duration spaceflight. The investigators have outlined several potential concepts for preadapting the human LVOR in astronauts. This group's participation in the JSC Neurological Function IPT has helped formulate plans for more comprehensive postflight oculomotor examinations of crewmembers by flight surgeons. In the areas where they have been working, Dr. Shelhamer's group has moved our understanding of these problems from Countermeasure Readiness level 1 to Level 2.

Visual Orientation in Unfamiliar Gravito-Inertial Environments. (C.M. Oman, Massachusetts Institute of Technology and 4 co-investigators.) What visual cues do astronauts rely upon to maintain their spatial orientation and sense of where they are relative to other objects and places ? Why is living in a three dimensional structure like the MIR space station disorienting ? What visual cues normally provide us with cues to the gravitational vertical on Earth ? Does inadvertent use of these same cues cause disorientation when astronauts live in weightlessness ? Does 1-G training in simulated 0-G real or virtual environments improve spatial orientation, spatial memory and task performance ? Using tumbling room and mirror bed devices, the group showed that compelling visual scenes can produce much larger illusions of subjective tilt than had previously been thought. For example, many supine subjects feel erect which viewing an environment which is visually upright with respect to their bodies, resulting in an interesting "levitation" sensation if subjects elevate their arms. The strength of tilt illusions also depends on the gravitational "polarity" of objects in the scene. This attribute depends both an object's usual orientation in 1-G everyday life, and on its means of apparent physical support. A correlation between tilt illusion susceptibility and age was found. Results on the polarity of objects in a visual scene can be used to update and extend NASA human factors standards on spacecraft visual verticals, and also suggest specific countermeasures. For example, placing pictures of "gravitationally polarized" objects on walls could make older people less prone to falls, and astronauts less prone to visual reorientation illusions. In a collaborative project with the Cardiovascular team, the investigators found that if a scene is sufficiently polarized and realistic, e.g. produced using an inclined mirror, the resulting tilt illusion can produce transient cardiorespiratory changes, further evidence of visual/vestibular-autonomic coupling. The team has also studied 3D spatial memory in a task analogous to that confronting astronauts in the node module of a space station. Though the experiments were performed in 1-G, spatial memory and learning was not strongly dependent on the gravitational orientation of the subject, nor on whether a virtual or real training environment was used. Instead, performance depended on the mental image and mnemonic strategies used, and correlated with performance on conventional 2 and 3 dimensional mental rotation ability, and visual field dependence. Training the subjects on generic 3D orientation strategies was found to help. Skills acquired were shown to transfer to a second environment, and were retained for at least several weeks. The training method could be used as the basis of a generic preflight spatial orientation training procedure for astronauts. Lastly, this group has studied the limbic coding of 3D orientation in 1-G and in an animal model in parabolic flight. Head direction cells in the anterior thalamic nuclei of rats retained normal visual environment-referenced responses in zero g and hypergravity when on the floor or walls. When crawling on the ceiling, directional specificity was frequently lost. However, when the animal was on the ceiling in 0-G, reversals in cell preferred response direction were found. The investigators believe this phenomenon is the neural correlate of visual reorientation illusions described by humans in analogous situations. The finding helps explain rat place cell results obtained on the Neurolab Spacelab mission. The team has succeeded in defining a scientific basis for preflight visual orientation training, the main goal of the project. In terms of countermeasures readiness, Dr. Oman's team has moved several concepts from Level 2 through Level 5.

Advanced Techniques for Assessment of Postural and Locomotor Ataxia, Spatial Orientation, and Gaze Stability. (C. Wall, Massachusetts Eye and Ear Infirmary and 6 co-investigators) Astronauts returning from long duration missions typically report postflight problems with standing, walking, and gaze stabilization. The longer the exposure to weightlessness, the more profound and long lasting the postflight deficits usually are. This group has developed a number of quantitative methods for measuring gaze, head and body stability during normal and perturbed locomotion. One technique, "Ideal Trajectory Analysis" (ITA) yields a measure of kinematic deviation from an ideal sinusoidal body center of mass trajectory. Analyzing data from subjects performing a repeated "stepping up" task, the investigators can statistically discriminate normals and patients. ITA has been used to evaluate the effects of vestibular rehabilitation in patients, and may also prove useful for assessing the performance and rehabilitation of returning astronauts. The project has also better defined the

relationship between head motion and walking speed, and between eye motion and visual target distance. During normal walking, the head pitches in phase with gait so it aimed consistently at a point called the "Head Fixation Distance", located about one meter ahead, and independent of gaze fixation distance. This is partly the result of a linear vestibulo-collic reflex (lVCR), responding to the vertical kinematic component of walking. When looking at a point far away during normal walking, the angular vestibulo-ocular reflex provides appropriate ocular stabilization. Looking near invokes the vertical LVOR, which may be affected by spaceflight. The angular vestibulo-collic reflex is dominant at low walking speeds and when viewing objects far away, and the lVCR is dominant at the higher optimal walking speeds, and when viewing near targets. Astronauts often report postflight difficulties while walking around corners, so the group has studied the eye, head and body response in normal subjects while walking straight as compared with walking a curved path, and showed how responses align with the resultant gravito-inertial acceleration. The group has also measured responses to perturbed gait by measuring the response of different body segments (head, trunk, legs) to a controlled mechanical perturbation of the foot after heel strike. Vestibular patients require several more steps to recover than normals. Several of these test techniques are now well defined, and are candidates for evaluation as postflight neurovestibular assessment tools to assess the return of normal function in astronauts. Dr. Wall's team has moved these from Countermeasures Readiness Level 1-2 to Readiness Level 5.

Looking to the future, several of these promising countermeasures concepts are near the point where they can be transitioned to JSC for further evaluation and development. In the important area of context-specific preadaptation training development, much work remains to be done. We hope to be able to expand the program to include several new areas of emphasis, including artificial gravity, postflight neurovestibular rehabilitation, and improved anti-motion sickness drugs.

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I. PROGRAM RESEARCH ACCOMPLISHMENTS

A. Original 1997 Research Objectives

The NSBRI neurovestibular adaptation research program provides a framework for focused research intended to rapidly develop scientifically based countermeasures for astronauts and vestibular patients. In developing the program research strategy for our 1996 proposal to establish NSBRI, we took a "top down" approach, and defined five priority research questions. The questions reflected the issues defined by the NASA/NIH Workshop on Vestibular Autonomic Regulation, a NASA Vestibular Countermeasures Task Group, and discussions with our External Advisory Committee, European and NASA-JSC investigators. The number of important questions in space neurovestibular research is certainly not limited to five, but we were guided by the number of projects that could reasonably be funded.

1. Can vestibular reflexes be preadapted (conditioned) in context specific ways, so astronauts can rapidly transition between alternative environments, i.e. between terrestrial, orbital, artificial, and planetary gravity, with minimal performance impairment?

2. Does 1-G training in simulated "agravic" environments (e.g. using neutral buoyancy or virtual reality techniques) improve orientation and navigation ability and task performance?

3. What causes the profound impairment of posture, gaze stability, and locomotion seen in vestibular patients and some - but not all - returning astronauts, and how can it be quantified?

4. Why do certain clinical vestibular rehabilitation techniques work in patients, and can these strategies be adapted for astronauts?

5. How do the neurovestibular and neuromuscular systems interact in 0-G ? Can mathematical models predict the effects of changing the real or simulated gravitational environment on whole-body movement control ?

In June of 1997, our team proposed a set of five specific projects, each targeting one of these questions. A peer review was conducted by the NSBRI Board of Scientific Counselors. Based on the results, NSBRI funded projects in the first three areas, described below. Proposals addressing the remaining two questions, vestibular rehabilitation and neuromuscular control were not funded. Six months later, a one year interdisciplinary "synergy" project with the Cardiovascular team was also initiated.

The relationship of the three funded core projects to the overall program goals is shown schematically in Fig. 1. The administrative organization of the three project, and their relationship to other the discipline teams is shown in Fig.2.

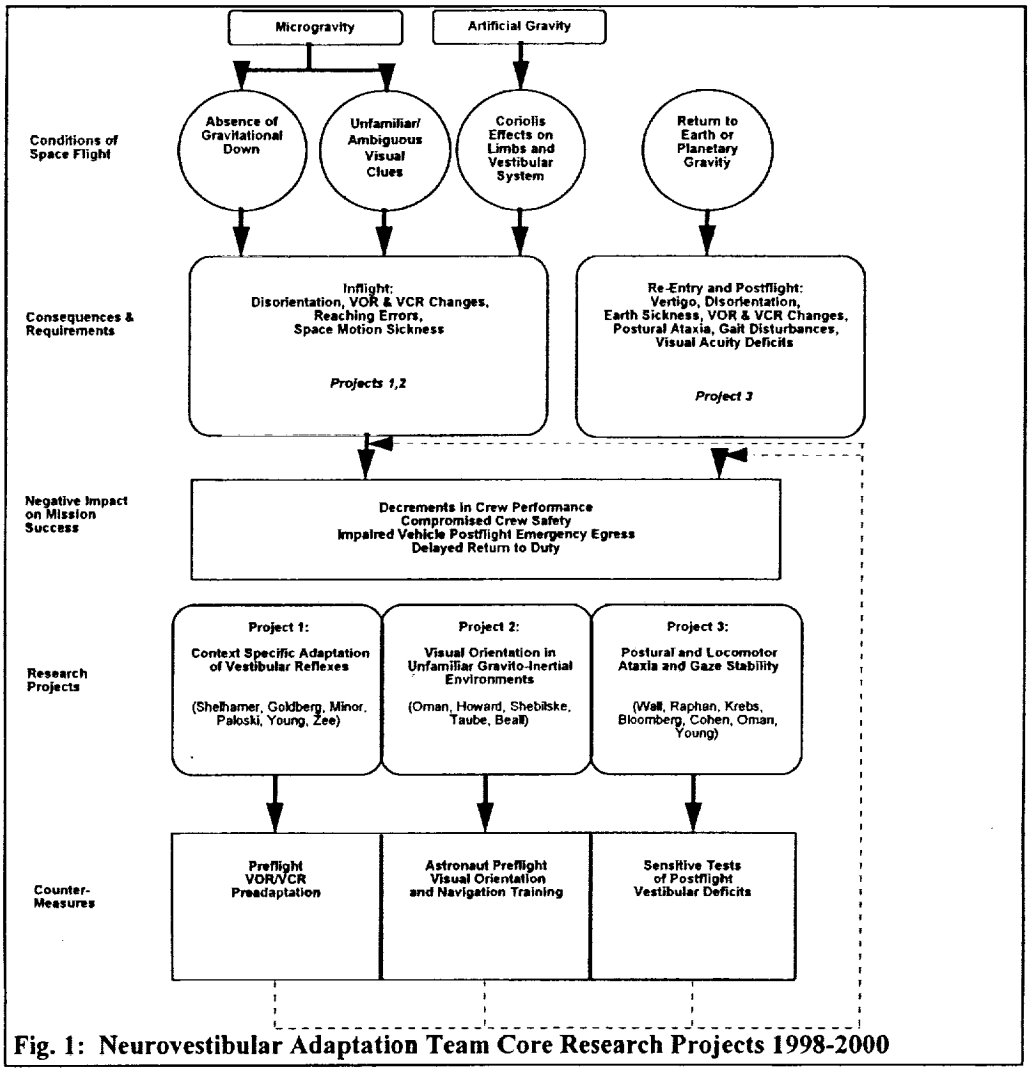


Fig. 1: Neurovestibular Adaptation Team Core Research Projects 1998-2000

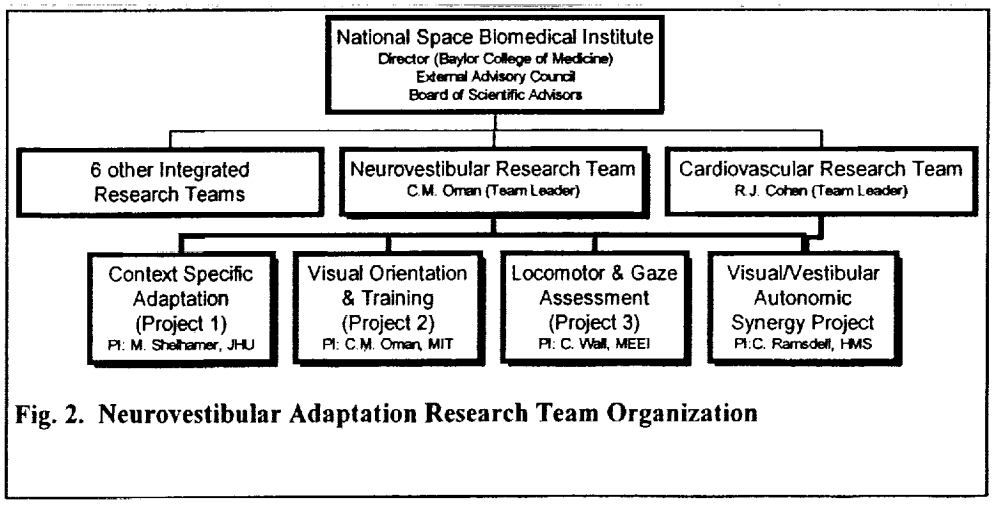


Fig. 2. Neurovestibular Adaptation Research Team Organization

Dr. Shelhamer's project addressed context specific adaptation of the VOR, VCR, and saccadic systems. It teamed Drs. Shelhamer, Zee and Minor's human and animal research facilities at JHU with Dr. Young and his lab at MIT, Dr. Goldberg at BCM, and Dr. Paloski's off axis rotator facilities at JSC.

Dr. Oman's project studied issues related to inflight spatial disorientation and spatial memory, with the objective of developing scientifically based preflight spatial orientation training countermeasures, using virtual reality techniques. The project built on the ongoing collaboration between Dr. Oman's VR group at MIT and Dr. Howard's vision team at York University in Toronto, and involves two investigators who were new to the space research arena, Dr. Taube of Dartmouth and Dr. Shebilske of TAMU. (Dr. Shebilske subsequently moved his research to Wright State University, where he became Psychology Dept. Chairman but remained active on the team.)

Dr. Wall's project goal was to develop improved methods for assessment of locomotion an gaze control, with potential application to the study of postflight deficits in astronauts. It combined the strengths of Wall's clinical research lab at MGH with those of Dr. Krebs' gait clinic at MGH, Dr. Oddson's neuromuscular lab at BU, Dr. Young's vestibular sled lab at MIT, Dr. Cohen's vestibular rehabilitation lab at BCM, and Dr. Bloomberg's lab at JSC.

Dr. Ramsdell's synergy project goal was to understand how cardiovascular changes measured with now widely used neck barocuff method compare with those obtained using the newer MIT developed Cardiovascular System Identification technique, and to see whether visual cues can elicit changes in cardiorespiratory regulatory responses. It teamed Dr. Ramsdell with Dr. Yelle of JSC, Dr. Wood of Baylor, and Drs. Oman representing the Neurovestibular team. Experiments were conducted at JSC, but utilized a technique for manipulation of the perceived gravitational vertical developed by Dr. Howard of the Neurovestibular team.

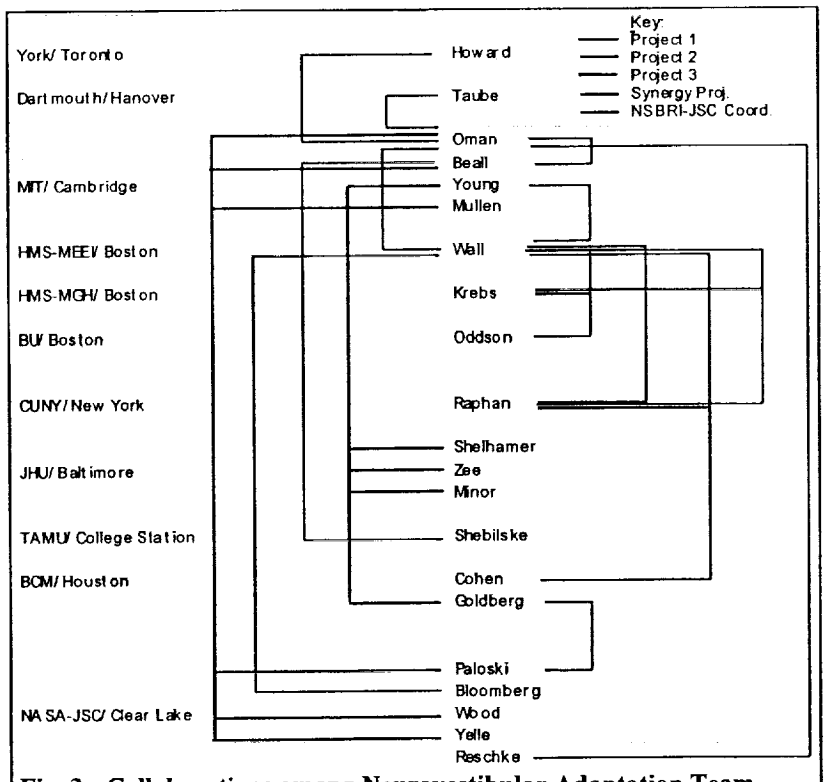


Fig. 3: Collaborations among Neurovestibular Adaptation Team members. Grouped according to geographic locus and institution.

Our team approach was patterned on the successful NIH Program Project Grant (PPG) model. However we extended the multi-investigator aspect of the PPG within as well as between our projects, because of the interdisciplinary nature of the research questions we addressed. The program provided our research team - numbering 21 investigators from 11 institutions - access to unique resources available at NSBRI consortium institutions, affiliates, and NASA centers, and NASA's KC-135 aircraft. An intellectual roadmap of the collaborations established is shown in Fig.3 at the left.

To maintain communications between the different components of the team, the Principal Investigators held telecons amongst themselves and with their CoInvestigators at frequent intervals. All team investigators participated in both NSBRI annual retreats (June, 1998; January 2000), the NASA Biomedical Investigators Workshop (January, 1999), and a team research strategy retreat held in Baltimore in December, 1998. Eight team members participated in a research strategy planning workshop in November, 1999.

Dr. Wall as served as Associate Team Leader, and assisted Dr. Oman with team coordination. Dr. Millard Reschke of JSC served as the official point of contact between NASA and the Team Leader. Drs. Shelhamer , H.Cohen, and Oman served as the team's liasion to the NASA JSC Neurological Integrated Projects Team, led by Dr. Jonathan Clark, a NASA Flight Surgeon. Neurovestibular Adaptation team investigators also served on the NSBRI Human and Animal Use (Dr. Taube), Data Sharing (Dr. Shelhamer), and Technology Team (Dr. Wall) advisory committees.

In addition to our core Neurovestibular Team program, in 1999, NSBRI and NIH-NIDCD set up a complementary program of basic neurovestibular research grants. Five investigations were selected. These were:

- D.E. Angelaki, Washington University School of Medicine, "Neural Mechanisms of Vestibular Adaptation"
- W.M. King, University of Mississippi Medical Center, "Signal Processing and Adaptation in Central Otolith Pathways"
- D.M. Merfeld, Massachusetts Eye and Ear Infirmary, "Decoding of Gravireceptor Cues, Including Adaptive Changes.
- J.L. Raymond, Stanford University, "Vestibular and Visual Control of Eye Movement"
- S.H. Seidman, University of Rochester, "Plasticity In the Vestibuloocular Reflexes and Perception"

These investigators participated in the January, 2000 NSBRI retreat. In addition to their ongoing NIH-NSBRI basic grants, several have proposed to collaborate in new core program projects.

Our team actively supported NSBRI's public education and outreach activities. General information about our research programs is available through the main NSBRI Web Site (www.nsbri.org). We also make more detailed information available to the public through our own neurovestibular adaptation team web site (http://mvl.mit.edu/Neurovestibular/Pages/home.html). During the past year, we worked with NSBRI's Education and Public Outreach Program through the Harvard Medical School Teacher Institute to complete "Cecilia's Story", a case based teaching unit on neurovestibular problems for high school students and teachers. The teacher's guide is available on the web. (www.nsbri.org/Education/High_Act.html). The team's research has been featured in several newspaper and magazine articles, and radio spots and television and streaming video productions on space biomedicine.

B. Mars Mission Critical Path Roadmap Project and Timeline for Neurovestibular Research

When NSBRI was created in the spring of 1997, the Team Leaders and management met with NASA Administrator Dan Goldin. Mr. Goldin emphasized the importance of the new Institute's role in accomplishing basic research leading specifically to Mars mission countermeasures development. Shortly afterward, the NASA Life Sciences Directorate initiated a "Critical Path Roadmap Project (CPRP)" to define and prioritize the research questions which needed to be resolved before a commitment to send humans to Mars is made. Drs. Oman, Wall, and Reschke represented the Neurovestibular team in the first phase of CPRP project activities. Dr. Oman has taught an MIT seminar on the case for human planetary exploration, and was familiar with the NASA Mars Design Reference Mission and other alternatives. Members of our team discussed the issues with astronauts, flight surgeons, mission designers, and other researchers. The nation could commit to a human Mars exploration mission as early as the next decade - or whenever a national consensus is reached that it is time to go - and development of appropriate large

launch vehicles has begun. To maintain support for the program, the first human landing ought to be accomplished within 4-8 years thereafter. In the interim, NASA is focussing on ISS missions to resolve the biomedical and operational issues associated with long duration spaceflight, and robotic exploration to define scientific goals and strategy for such a mission. The ISS provides an important opportunity to validate potential 0-g biomedical countermeasures, but it is not an unlimited resource. Over the next decade, only 60 crewmembers will be available as human subjects in orbit. Much of the process of countermeasures development will have to take place on the ground.

From the perspective of neurovestibular research planning for a Mars mission, key concerns are:

1. Fully automatic Mars landing capability will be required for unmanned vehicles and therefore likely be available for manned ones. Manned vehicles will be "piloted", but the landings will likely not be manually flown to touchdown, as the Shuttle or Apollo lunar landings were, but will instead monitored, in a fashion analogous to an airliner's Category III automatic landing. The neurovestibular challenge comes because crewmembers who have been weightless for more than several days typically experience vertigo when they reencounter a changing gravito-inertial acceleration, and particularly when they make head movements. The accelerations encountered during Mars aerobraking or during a parafoil landing in an Earth return vehicle are significant stimuli. Cockpits should be designed so that pilots do not have to make head movements to accomplish their supervisory tasks. Even so, it may not be possible to completely eliminate the disorienting stimulus. Pilots prefer not to take anti-motion sickness drugs because of their potentially sedating effects, and because they know the drugs do not prevent vertigo and disorientation, and have limited effectiveness against nausea and vomiting.

2. After landing on Mars or Earth, crewmembers may require several days to adapt, unless countermeasures can be found which will accelerate this process. Shuttle crewmembers typically experience oscillopsia and mild ataxia after 1-2 week flights. MIR crewmembers who have flown for 3-5 months have experienced more profound symptoms, which has made them vulnerable should they suddenly be called upon to escape from the vehicle. Long duration crewmembers who have tried running on a treadmill during the first day after return from orbit been unable to run several hundred yards. It is reasonably clear that this disability is primarily involves balance and motor control, and is not simply the result of muscular deconditioning. Altered vestibular function may also be contributing to postlanding orthostatic hypertension. Postlanding disorientation usually results in motion sickness symptoms, and mild to moderate cognitive impairments.

3. Vulnerability to space sickness and disorientation is not limited to the first several days of a trip to Mars. The voyage to and from Mars will be punctuated with occasions where there are significant vehicle accelerations (orbital change engine firings), dockings and transfers of personnel and equipment between vehicles, and other activities requiring body acceleration and sustained physical movement. There is a likelihood that disorientation, ataxia and space motion sickness will re-arise in some individuals when physical activities or g changes are re-initiated. Reappearance of space motion sickness has been seen in the Russian program 2-3 months into spaceflight. Crewmembers on MIR missions have reported difficulties with visual orientation and spatial memory, even after weeks in flight, and some have found they are vulnerable to EVA acrophobia, a disabling fear that they will fall off the vehicle. Vomiting in a space suit during EVA, particularly while in 0-g, is unpleasant and probably dangerous, and makes the suit unusable.

4. Artificial gravity (AG) is being reconsidered for use on ISS and Mars Missions, because using it would provide a common solution to problems identified in the bone, muscle, cardiovascular, and neurovestibular areas. Concepts range from rotating spacecraft (e.g. von Braun torus, or two spacecraft connected by a tether) and rotating cabins (e.g. Kubrick's "2001") to achieve sustained artificial G via centrifugal acceleration, to short radius centrifuges located within the spacecraft cabin for intermittent exposure. The latter would toallow crewmembers to expose themselves to a preadapting "g – bath". How much "g" is required, and for how long is as yet unknown. The engineering of short radius centrifuges is simpler than rotating an entire spacecraft cabin. Preliminary design work is underway at JSC for a short radius centrifuge for a Spacehab shuttle research mission several years from now. From the neurovestibular point of view, AG is a double-edged sword: Many expect AG can be successfully used in 0-g to pre- or dual-

adapt astronauts to Martian and Earth's gravity. On the other hand, head movements are made out of the plane of rotation produces an un-natural flow of endolymph in the semicircular canals of the vestibular system, and a strong illusory tumbling sensation about an axis perpendicular to the head movement, known as the "vestibular Coriolis response". The direction of the illusion depends on which way the person is facing relative to the rotational motion. Experiments in 1-g laboratories, on Skylab and in parabolic flight have shown that when a g field is present, out-of-rotation-plane head movements soon produce motion sickness in the large majority of individuals. The provocative nature of the head movements depends on the g level the otolith organs experience. Tests in 0-g with the head located on the axis of rotation so the otoliths are weightless have shown that out of rotation plane head movements are not very provocative. However, if the head is positioned off the axis of rotation in an spacecraft centrifuge, in order to provide an adapting centrifugal g stimulus to the otoliths, head movements are expected to be provocative unless somehow limited to the plane of rotation, to eliminate the Coriolis reaction. There is reason for optimism, but if AG is to be utilized, defining the limits of human neurovestibular "g-dose" duration requirements and adaptability is clearly a priority.

5. It is conceivable that long duration exposure to 0-g will induce pathological changes in the adult vestibular end organs, particularly the otolith organs. We know that the body looses calcium at a regular rate. The otoconial crystals which form the seismic mass in our inner ear biological linear accelerometers are composed of calcium carbonate, and undergo regular turnover, and conceivably their rate of formation and destruction may be affected by changes in whole body calcium. Gravity is also thought to normally play a role in mechanically confining loose otolith crystals to the utricular chamber. Russian and US animal experiments have so far produced equivocal or conflicting results as to whether otoconial changes occur. Changes have also been reported in the ultrastructure of the vestibular sensory epithelia, although the functional significance of this finding remains unclear. Direct evidence for permanent endorgan pathology in humans is lacking, since it requires postmortem histological studies. Indirect evidence could come from postflight tests of otolith mediated reflexes in long duration crewmembers, but such tests have not yet been accomplished, and arguably the appropriate clinical tests are not yet sufficiently diagnostic. There is also some concern about the effects of the space radiation environment on the CNS, and that neurovestibular pathways may be susceptible to damage.

To support the CPRP effort, we summarized the neurovestibular risks of a Mars mission as shown in Figure 4 below, and detailed at (http://criticalpath.jsc.nasa.gov/risks.asp?DiscMode=D009). The CPRP methodology asked each discipline to define initiating events, predisposing factors, the individual risks themselves, and their consequences:

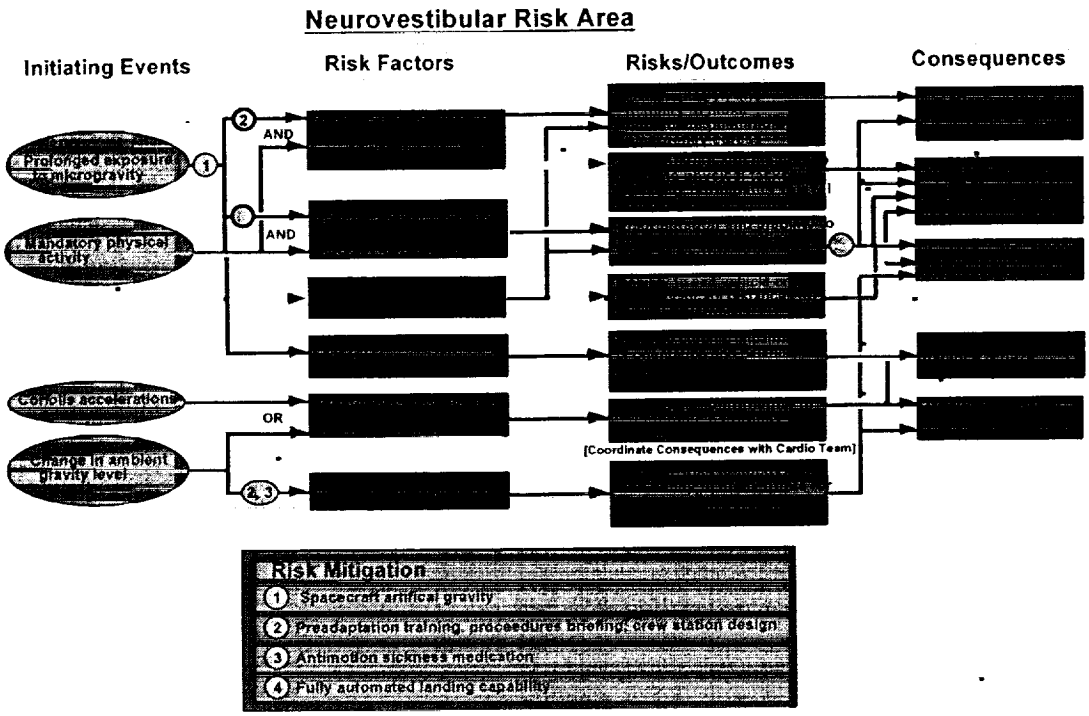


Fig. 4 Schema developed for Critical Path project assessment of Neurovestibular Adaptation risks.

NSBRI defines the term "neurovestibular" broadly, so our research addresses not only on potential changes in the vestibular end-organs themselves, but on adaptive changes in the CNS centers which control balance and eye movement stabilization reflexes, and autonomic and emetic reflexes. As a result, our list of specific risks and risk factors was initially a very lengthy. Ultimately, we chose to reduce the list of risks to five, and provide both plain language and specific pathophysiological definitions.

The exercise of defining risks and their potential impact on mission operations in each discipline has certainly been worthwhile. However, the methodology we used does tend to suppress the true complexities and uncertainties of many problems. For example, no effort was made to assess confidence in assumptions about risk probabilities, or anneal the conclusions by defining nominal and off nominal mission scenarios. Neurovestibular issues are of particular importance in off nominal and emergency situations. The formal quantitative failure analysis methods originally developed in the aerospace community were not utilized by CPRP, though some might assume so given the title of the project. Although the first stage definition of risks within disciplines was done by discipline specialists, and was an open process, the second stage classification of the relative risks across disciplines is more difficult and has been done by a second, smaller group using a less precise methodology. Some sort of critical path analysis is necessary, and we have tried to assist the process. However, we remain concerned the conclusions of the CPRP results could be over- interpreted or misused by management and advisory committees unless they are aware of the limitations and biases of the underlying two stage process.

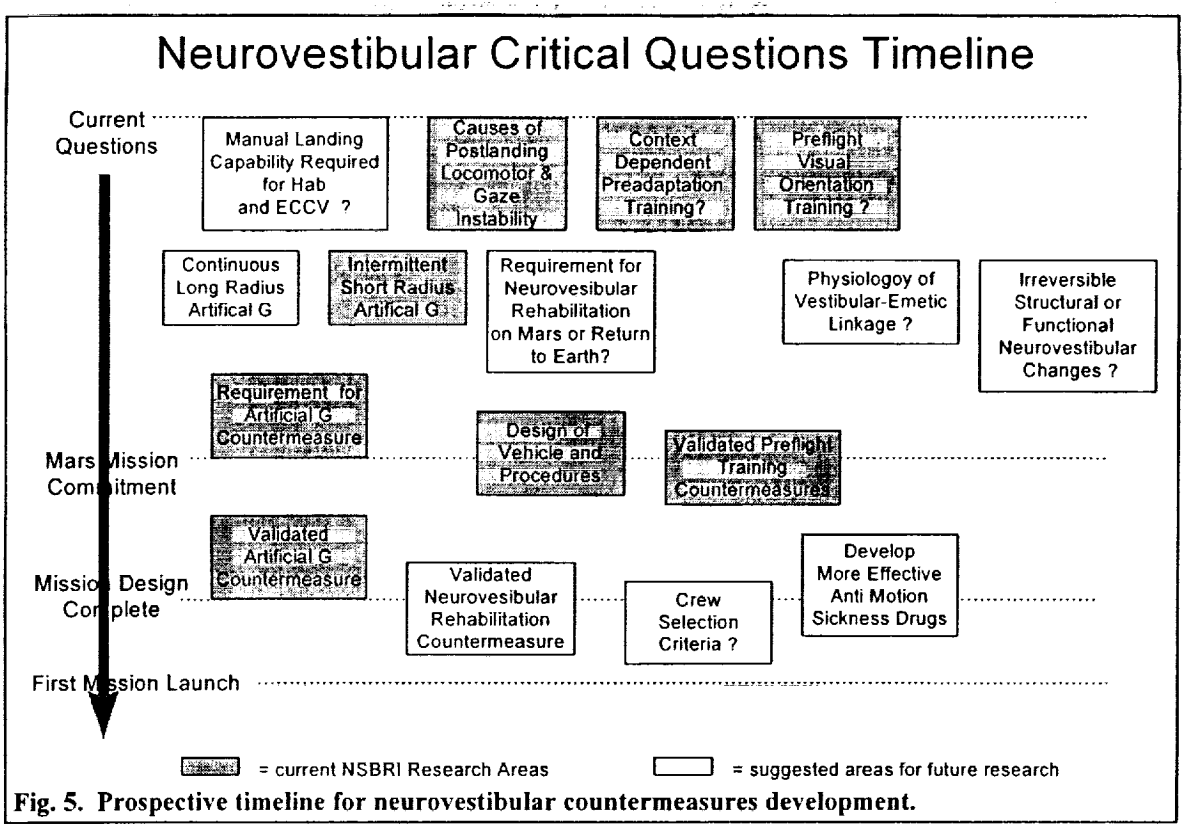


Fig. 5. Prospective timeline for neurovestibular countermeasures development.

Based on the CPRP effort, and the recommendations of a report released by the Committee on Space Biology and Medicine (http://www.nas.edu/ssb/csbmmenu.htm) we mapped our research goals and current project portfolio into a timeline (Fig 5.). We concluded that although the current research team's work was focussed in high priority areas, a broader effort was required. In addition to our current projects, additional efforts need to be started soon to develop postflight neurovestibular rehabilitation techniques, scientifically based anti-motion sickness drugs and methods for assessing their side effects, long and short radius artificial G. More needs to be done to define the possibility of deficits in oculomotor gave control and irreversible functional or structural neurovestibular end organ changes in crew members who have flown for many months or years in space. NSBRI and NASA need to complete the definition and implementation of the countermeasures transition process, and make major decisions regarding the use of artificial gravity, and the human's role in physical and time critical mission phases as soon as possible. These arguments, and those from other disciplines, formed part of the basis for the major augmentation of NSBRI's budget proposed as part of NASA's Bioastronautics initiative.

C. NSBRI Neurovestibular Program Goals

To anneal our conclusions, and to establish priorities for NSBRI neurovestibular research in the 2000-2003 timeframe, we convened a "Neurovestibular Adaptation Workshop" in Houston in November of 1999. Twenty six neurovestibular specialists participated. Dr. Oman chaired the meeting, and the other two project PIs attended, but nineteen of the participants came from outside the Core team. Several astronauts spoke about their experiences, including Dr. Daniel Barry, a physician who had flown to ISS several months previously. We reviewed the Committee on Space Biology and Medicine recommendations, the CPRP risks and the rationale for them, and the goals of our program, which can be most practically stated as to accomplish research leading to countermeasures that ultimately will allow crewmembers to:

- Avoid disorientation
- Meet physical requirements of emergencies

- Treat motion sickness without side effects
- Safely control vehicles and systems.

We defined nine research themes, and a set of associated research questions to go with them. The themes are:

1. Adaptive Generalization and Context-Specific Adaptation
2. Artificial Gravity
3. Visual (multisensory) orientation, navigation and spatial memory
4. Drug countermeasures
5. Postflight locomotion and gaze assessment
6. Neurovestibular rehabilitation
7. Vestibular effects on autonomic function
8. Effects of weightlessness, stress, isolation, immobilization and diet on vestibular function.
9. Potential mechanisms for and diagnosis of irreversible neurovestibular changes.

The detailed research questions are listed in the report of the Workshop, which is included as Appendix A. Broad research areas which potentially cut across multiple research domains were also identified, including artificial gravity, improved drug delivery and monitoring systems, and the need to optimally integrate inflight and postflight assessment and rehabilitation efforts across disciplines was noted.

These lists of themes, risks and research questions formed the basis for NSBRI's Research Announcement 99-00-01 "An Opportunity to Participate in the Core Research Program of the National Space Biomedical Research Institute - Expansion of Current Research Teams" (downloadable from: http://www.nsbri.com/nra/nra.cfm)

In the Spring of 2000, several new NSBRI discipline teams were created. Our Neurovestibular Adaptation research overlaps to some degree with the domain of the Neurobehavioral and Psychosocial team (particularly in the area of performance deficits and assessment), Nutrition, Fitness and Rehabilitation (particularly in the area of rehabilitation of balance function), and Integrated Human Function (since mathematical modeling has a long tradition in the neurovestibular discipline, dating back to 1931.) An "Artificial Gravity" team was not created, reflecting a consensus that AG is a countermeasure that crosscuts many of the discipline teams, and that NSBRI would be better served by a horizontally structured AG program. Taking a first step in that direction, in the spring of 2000, NSBRI convened an interdisciplinary group to review potential design concepts for a short radius centrifuge for a shuttle/Spacehab flight, and to define and prioritize the associated AG research questions.

One result of the CPRP planning process was the recognition that we lacked detailed descriptions of the neurological problems associated with spaceflight, particularly in the neurovestibular area, and statistics on the relative incidence. Data is available from short duration Shuttle flights in papers written by flight surgeons and Spacelab investigators. However, only anecdotal data has so far been available from the NASA-MIR and early ISS flights. In order to compile the necessary data, Mr. Jason Richards is currently spending 3 months at JSC on a NSBRI internship in the office of Flight Surgeon Dr. Jonathan Clark. Mr. Richards was formerly a graduate student working in Dr. Oman's MIT lab, and is working with Dr. Clark to review crew reports from long duration missions, formulate an appropriate taxonomy of the neurovestibular problems in long duration spaceflight, and compile basic statistics on incidence and severity. Dr. Tom Marshburn, a NASA flight surgeon who participated in NASA-MIR, and Dr. Oman are advising on the project.

In June, NSBRI received 16 proposals in response to Research Announcement 99-00-01. Proposals were reviewed by an independent peer-review panel. Dr. Oman was reappointed Team Leader, and two Associate Team Leaders were officially designated, Dr. Wall (who had served informally in this capacity) and Dr. Bernard Cohen, an experienced neurovestibular researcher and clinician from Mt. Sinai Hospital in

New York. Those in the competitive range were evaluated by the Team leadership, with assistance from the NSBRI External Advisory Council. We hope to complete our current efforts and expand in several important new directions, but as of this writing (9/30/00) the NASA FY2001 budget has not been finalized, so the full scope of NSBRI's new research team portfolios has not yet been announced.

II. RISK REDUCTION ACHIEVED BY PROGRAM

NASA and NSBRI define countermeasures readiness in terms of three phases, and nine levels:

Basic Research

1. Phenomenon observed and reported, problem defined.
2. Hypothesis formed, preliminary studies to define parameters, demonstrate feasibility.
3. Validated hypothesis, understanding of the scientific processes underlying the problem.

Countermeasure feasibility and development

4. Formulation of countermeasures concept, based on understanding the phenomenon.
5. Proof of concept testing and initial demonstration of feasibility and efficacy.
6. Laboratory/clinical testing of potential countermeasure in human subjects to demonstrate efficacy of concept for a specific problem.

Ground evaluation and flight validation

7. Integrated evaluation with human subjects in controlled laboratory conditions simulating operational space flight environment.
8. Validation with human subjects in actual operational space flight to demonstrate efficacy and operational feasibility.
9. Flight implementation

During the first 3 years, NSBRI has not undertaken flight research. Most Countermeasures development has been in the 1-7 range. The accomplishments of the three current projects are thoroughly summarized and detailed in individual Final Reports, which will not be repeated here. These reports also discuss implications for future research. How successful were our projects in achieving their goals ? How far did each of the three core projects move our discipline in terms of countermeasures readiness ?

Project 1: Context-specific adaptation of gravity-dependent vestibular reflex responses.
(M.J. Shelhamer, et al). Adaptation to weightlessness and readaptation to Earth's, planetary, or artificial gravity requires adaptive changes in many of the body's sensory-motor reflexes. Normally, adaptation to a series of new environments occurs sequentially, and crew members experience some degree of disorientation and ataxia until the process is complete. Can vestibular reflexes be pre-conditioned in context specific ways, so that adaptation occurs much more rapidly, or so the appropriate pre-adapted responses are immediately invoked ? If so, what are the essential characteristics of the conditioning stimulus ? This basic human and animal research project succeeded in obtaining fundamental evidence on several types of context-specific adaptation in both human and animal subjects. It was shown that g-level and eye position can be used as a context cue for changing the size of saccadic and smooth pursuit eye movements, and that saccadic adaptation can persist for many months. G-direction can provide a context cue for adapting the Linear Vestibulo Ocular Reflex (LVOR). In both cases, the evidence suggests that the more relevant the context cue is to the response being adapted, the more effective it is. Adaptation of the Vestibular Coriolis oculomotor response in a rotating environment – and retention of it - is of particular importance to our artificial gravity research theme. It was shown that three ten-minute periods produced measurable reduction of inappropriate eye movements, and adaptation was retained one week later. Some support for context specificity was found: subjects did not experience motion illusions when head movements were made in a non rotating environment. In other experiments, it was shown that the human vestibulo-collic reflex adapts much more quickly to artificial increases in head inertia after several practice sessions on consecutive days. Some progress was made using primate models to understand the underlying physiology of adaptation, though not as much as originally planned due to various technical problems: LVOR adaptation was found to be specific to the frequency used. There was no correlated

change in static ocular torsion resulting from head tilt. Floculectomy permanently reduced the LVOR, even after the angular VOR had recovered. Human cerebellar patients show comparable deficits. The investigators note that the "0-g flashbacks" anecdotally reported by some cremembers are consistent with the notion of dual-state adaptation. evidence They have also pointed out that cerebellar adaptation failure might account for a number of the oculomotor problems reported in the Russian literature after long duration spaceflight. The investigators have outlined several potential concepts for preadapting the human LVOR in astronauts. This group's participation in the JSC Neurological Function IPT has helped formulate plans for more comprehensive postflight oculomotor examinations of crewmembers by flight surgeons. In the areas where they have been working, the team has moved our understanding of these problems from Countermeasure Readiness level 1 to Level 2.

Project 2. Visual Orientation in Unfamiliar Gravito-Inertial Environments.
(C. Oman, et al). Astronauts report inversion and visual reorientation illusions, EVA acrophobia, and 3D spatial memory problems when moving between modules which can impair performance, and sometimes trigger space motion sickness. The design of ground based simulators and training experiences could undoubtedly be improved if we could better understand what visual scene attributes determine perceived orientation in 3 dimensions, the role of experience in 3D spatial memory, and more about the physiology of our orientation sense in 3 dimensions. This team has shown that compelling visual scenes can produce much larger illusions of subjective tilt than had previously been thought. For example, many supine subjects feel erect which viewing an environment which is visually upright with respect to their bodies, and resulting in an interesting "levitation" sensation if subjects elevate their arms. The strength of tilt illusions also depends on the "polarity" objects in the scene. This attribute depends both an object's familiar orientation in 1-G, and its means of physical support. A correlation between tilt illusion susceptibility and age was found. Results could be used to update and extend NASA human factors standards on spacecraft visual verticals, and also suggest specific countermeasures. For example, placing pictures of "gravitationally polarized" objects on walls could make older people less prone to falls, and astronauts less prone to visual reorientation illusions. If the scene is sufficiently polarized and realistic, e.g. produced using an inclined mirror, the investigators found the tilt illusion can produce transient cardiorespiratory changes, further evidence of visual/vestibular-autonomic coupling to the cardiovascular regulatory system. The team has also studied 3D spatial memory in a task analogous to that confronting astronauts in the node module of a space station. Though the experiments were performed in 1-G, spatial memory and learning was not strongly dependent on the gravitational orientation of the subject, nor on whether a virtual or real training environment was used. Instead, performance depended more on the mental image and mnemonic strategies used, and correlated significantly with performance on conventional 2 and 3 dimensional mental rotation ability, and visual field dependence. Training the subjects on generic 3D orientation strategies was found to help. Skills acquired as a result of the training transfer to a second environment, and are retained for at least several weeks. The training method could be used as the basis of a generic preflight spatial orientation training procedure for astronauts. Lastly, this group has studied the limbic coding of 3D orientation in 1-G and in an animal model in parabolic flight. Head direction cells in the anterior thalamic nuclei of rats retained normal visual environment-referenced responses in zero g and hypergravity when on the floor or walls. When crawling on the ceiling, directional specificity was frequently lost. However, in 0-G, reversals in cell preferred direction were found with the animal on the ceiling. The investigators believe this phenomenon is the neural correlate of visual reorientation illusions reported by humans in similar situations. The finding also helps explain rat place cell results obtained on the Neurolab Spacelab mission by McNaughton and colleagues. The team has succeeded in defining a scientific basis for preflight visual orientation training, the main goal of the project. In terms of countermeasures readiness, in at least two areas they have moved from Countermeasures Readiness Level 2 through Level 5.

Project 3. Advanced Techniques for Assessment of Postural and Locomotor Ataxia, Spatial Orientation, and Gaze Stability.
(C. Wall, et al.) In addition to inflight neurovestibular difficulties, astronauts returning from long duration missions typically report postflight problems with standing, walking, and gaze stabilization. The longer a crewmember flies, the more profound and long lasting the deficits usually are. This group has developed a number of quantitative methods for measuring gaze, head and body stability during normal and perturbed movement. One technique, "Ideal Trajectory Analysis" (ITA) yields a measure of kinematic deviation from an ideal sinusoidal body center of mass trajectory. Analyzing data from subjects performing a repeated "stepping up" task, the investigators can statistically discriminate normals and patients. ITA has been used to evaluate

the effects of vestibular rehabilitation in patients, and may also prove useful for assessing the performance and rehabilitation of returning astronauts. Floquet Muliplier analysis has also been evaluated. Computerized Dynamic Visual Acuity (DVA) testing of subjects walking on a treadmill invokes both gaze stabilization mechanisms. Preliminary data showing changes in DVA after acute vestibular nerve section, and differences in the horizontal linear vestibulo-collic reflex of chronic vestibular patients as compared to normals was obtained. (The investigator has since obtained other support for this work). Dr. Wall's group has also better defined the relationship between head motion and walking speed, and between eye motion and visual target distance. During normal walking, the head pitches in phase with gait so it aimed consistently at a point called the "Head Fixation Distance", located about one meter ahead, and independent of gaze fixation distance. This is partly the result of a linear vestibulo-collic reflex (lVCR), responding to the vertical kinematic component of walking. When looking at a point far away during normal walking, the angular vestibulo-ocular reflex provides appropriate ocular stabilization. Looking near invokes the vertical LVOR, which may be affected by spaceflight. The angular vestibulo-collic reflex is dominant at low walking speeds and when viewing objects faraway, and the lVCR at the higher optimal walking speeds, and when viewing near targets. Astronauts often report postflight difficulties while walking around corners, so the group has studied the eye, head and body response in normal subjects while walking straight as compared with walking a curved path, and showed how responses align with the resultant gravito-inertial acceleration. The group has also studied responses to perturbed gait by measuring the response of different body segments (head, trunk, legs) to a specific mechanical perturbation of the foot. Vestibular patients require several more steps than normals, who recover in four steps and about 0.75 sec. Several of these techniques are now well defined, and candidates for evaluation as postflight neurovestibular assessment tools to assess the return of normal function in astronauts. Dr. Wall is working with Dr. J. Bloomberg of JSC to initiate transition. The project has moved these from Countermeasures Readiness Level 1-2 to Readiness Level 5.

APPENDIX

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Workshop on Human Vestibular Adaptation
Report and Recommendations

Houston, TX
1 December, 1999

BACKGROUND

NSBRI's neurovestibular adaptation research program supports research aimed at developing scientifically-based countermeasures against the vestibular problems associated with space flight: space motion sickness, disorientation, oculomotor deficits, postflight postural instability and gait ataxia. Neurovestibular problems typically arise first when astronauts transition from 1-G to 0-G, unfortunately just when their physical and cognitive performance is critical for mission success and safety. Similar problems are expected on exploration class missions when astronauts make the transition from 0-G to partial G, or from 0-G to an artificial gravity environment. During the Shuttle era, space neurovestibular research focused on understanding the effects of unweighting of the otoliths on the VOR, and predicting space sickness susceptibility. NSBRI's current early ISS-era neurovestibular research program is investigating context specific pre-adaptation, preflight visual orientation and 3D spatial memory training countermeasures, and improving our ability to assess postflight posture, locomotion and gaze control problems. Looking to the future, the program must expand to include several new areas of emphasis, including artificial gravity, postflight neurovestibular rehabilitation, improved anti-motion sickness drugs, and other areas defined below.

SCOPE

The NSBRI neurovestibular adaptation research program addresses five major space neurovestibular risk areas:

1. Disorientation and reduced performance on cognitive and physical tasks, including vehicle egress, especially during/after g-level changes (associated with acute spontaneous and head movement contingent vertigo, nystagmus, oscillopsia, saccadic errors, reduced dynamic visual acuity)

2. Impaired neuromuscular coordination and/or strength (gait ataxia, postural instability).

3. Impaired cognitive and/or physical performance due to spatial disorientation, motion sickness symptoms or treatments (including short term memory loss, reaction time changes, drowsiness, fatigue, torpor, irritability, ketosis) as a result of changes in g-level, or use of artificial gravity.

4. Autonomic dysfunction (including cardiovascular, respiratory, gastrointestinal, sleep and mood changes) which may be of vestibular origin.

5. Permanent impairment of orientation or balance function due to microgravity or radiation (causing chronic imbalance, gait ataxia, vertigo, chronic vestibular insufficiency, poor dynamic visual acuity)

The goals of the program are to develop countermeasures that ultimately will to allow crewmembers to:

- Avoid disorientation
- Meet physical requirements of emergencies
- Treat motion sickness without side effects

- Safely control vehicles and systems.

THEMES

Nine interrelated, countermeasures-oriented research themes currently define the scope of this program area:

1. Adaptive Generalization and Context-Specific Adaptation
2. Artificial Gravity
3. Visual (multisensory) orientation, navigation and spatial memory
4. Drug countermeasures
5. Postflight locomotion and gaze assessment
6. Neurovestibular rehabilitation
7. Vestibular effects on autonomic function
8. Effects of weightlessness, stress, isolation, immobilization and diet on vestibular function.
9. Potential mechanisms for and diagnosis of irreversible neurovestibular changes.

Currently research is being conducted at the cognitive, behavioral, system, organ, and cellular level, using quantitative techniques in both humans and animals. Molecular methods may also be appropriate. Much of the research is interdisciplinary, and involves collaborations between investigators at multiple institutions. Use of mathematical models as a research tool is encouraged.

NSBRI research is ultimately directed at the development of countermeasures. Basic research projects must plausibly lead in that direction. If specific countermeasures are proposed, they will ultimately have to be proven safe and practical, and their potential impact on other physiological systems understood. The dependent measures used to assess the effectiveness of the countermeasure must be defined. Individual differences in susceptibility to neurovestibular problems must be recognized.

The current postflight neurovestibular assessment is a 15 minute exam of neurological symptoms and signs, dizziness, motor performance and gait.

RESEARCH QUESTIONS

The following questions are provided to define the scope of the research area. This is not a complete list, and many questions are relevant to more than one theme.

Adaptive Generalization and Context-Specific Pre-adaptation

Can we enhance an individual's ability to adapt to multiple environments, for example through adaptive generalization ? What are the sensory-motor responses that must change in a functionally adaptive manner during prolonged space flight ? Does such adaptation does take place ? How can it be reliably measured ? Can these adaptive responses be trained to be context-specific ?

What is the evidence for and the physiological bases of oscillopsia, disorientation, ataxia, and reduced dynamic visual acuity reported by crewmembers, particularly while making head movements during re-entry and immediately postflight ?

To what extent can gravireceptor dependent motor responses be pre-adapted in context-specific ways, so astronauts can rapidly transition between 1-G and 0-G, 0-G and partial G, or 0-G and artificial G with minimal performance impairment or motion sickness ? How long does the pre-adaptation last ? Must the context cue be associated with active movement ?

How do countermeasures (e.g., artificial gravity, inflight exercise, or preflight training) affect adaptation rates and levels ? How do rates and levels associated with physiological (sensorimotor, autonomic,

emetic) adaptation to microgravity and 1/3 g on the Mars surface correlate with operational performance changes?

What are the appropriate spaceflight analog environments that can be used as test beds for evaluating neurological adaptation, adverse operational implications, countermeasures, and impacts of adaptation on other anatomical and physiological systems ?

Artificial gravity

What are the pros and cons of artificial gravity (AG) as a countermeasure against the effects of 0-G on neurovestibular function and on cognitive and physical performance? What are the advantages and disadvantages of large radius continuous AG vs. short radius intermittent AG and how are these influenced by mission duration and post-landing environment (Mars vs. Earth).
Can humans successfully adapt to working perpendicular to the angular velocity vector ?
How can transitions be eased ?
What is the maximum tolerable rotation rate for a given g level ? What is the best habituation schedule ?
What is the relationship between psychosocial factors and vestibular adaptation to altered gravity ?

Visual orientation, navigation and spatial memory

How do visual and nonvisual cues interact to influence human orientation perception and perceptual motor behavior ? Does 1-G training in simulated "agravic" real or virtual environments improve 3D spatial memory, and performance in orientation and navigation tasks ?

How do visual, vestibular, and haptic cues contribute to inversion illusions, visual reorientation illusions, extravehicular activity acrophobia, disorientation, and poor 3D spatial memory in 0-G ?

What is the physiological basis of inversion illusions, visual reorientation illusions, EVA acrophobia, disorientation, and 3D spatial memory problems in 0-G ?

How is the human sense of place and direction neurally coded in 0-G ?
Can preflight training techniques (e.g. virtual reality simulations) be used to alleviate these problems, and to evaluate emergency procedures ?

How can 0-G immersive teleoperation displays be designed to reduce disorientation and/or motion sickness ?

Anti Motion Sickness Drug countermeasures

Can improved anti-motion sickness drugs and delivery systems and dose and side effect monitoring systems be developed ? Drugs must be effective, and easily and safely used over days to weeks with minimal side effects, and must not impair adaptation. Ground based experimental models for evaluating 0-G pharmacokinetics and assessing the effectiveness of drug countermeasures are needed.

Postflight locomotion and gaze assessment

What causes the profound impairments of posture, gaze and locomotion stability in many returning astronauts (and in vestibular patients) and how can these be quantified ?

What causes the large differences in level of impairment observed among different people ? How do these differences correlate with physiological and operational performance changes?

How are the multiple, mutually dependent sensorimotor systems responsible for locomotion altered by exposure to spaceflight? For example, what is the role of the vestibulo-ocular, vestibulo-collic, and vestibulo-spinal reflexes, in 3D control of locomotion ?

How are target acquisition, smooth pursuit and saccadic mechanisms programmed during locomotion. How do oculomotor and gait control systems interact during locomotion and head turning ? How is this interplay affected by spaceflight?

Can long-term exposure to spaceflight impair sensorimotor plasticity ?
What roles do visual cues play in postflight locomotor control?

In an altered sensory environment, does motor control require increased cognitive resources, and does this multi-tasking impair performance ? Can a dual-task paradigm be used to monitor adaptation ?

What is the linkage between spaceflight induced changes in sensory-motor control and astronaut functional performance ?

What measures represent composite and global indicators of locomotor and/or gaze dysfunction after spaceflight. What measures are the most efficient and sensitive indicators of changes in locomotion and/or gaze, and their correlation with functional performance after spaceflight.

Neurovestibular preadaptation and rehabilitative countermeasures

Can preflight or inflight training, or sensory aids and prostheses and assessment techniques improve inflight orientation and gaze control and postlanding functional task performance and also postural and locomotor control ?
How can somatosensory information be used to assist adaptation ?

What are the relative contribution of neurovestibular adaptation, neuromuscular deconditioning, and orthostatic intolerance to postflight neuromuscular coordination, ataxia and locomotion difficulties ?

How does attention to a new sensory-motor task affect performance of a secondary task ?

Vestibular effects on autonomic function

What is the physiological basis of space motion sickness ? How does chronic space motion sickness (including sopite syndrome) affect mood, initiative, and interpersonal relationships ?

Does neurovestibular response to weightlessness impair postlanding cardiovascular regulation and contribute to orthostatic intolerance ? In what effective frequency range ? Can an effective countermeasure(e.g. AG) be developed to exploit this knowledge ?

Effects of weightlessness, stress, isolation, immobilization and diet on vestibular function.

How can changes in vestibular function due to weightlessness be distinguished from the normal responses to stress, isolation, diet and normal background physiological variability ? What countermeasures can be developed ?

Potential mechanisms for and diagnosis of irreversible neurovestibular changes.

How might very long duration exposure to 0-G or partial G cause irreversible (pathophysiological) changes in central or peripheral vestibular function or development or accelerate the normal aging process ? Would some individuals be more susceptible than others ? What is the potential time course ? How could such changes be reliably detected at an early stage ?

How does serum calcium homeostasis impact otoconial turnover ?

Workshop recommendations to NSBRI management:

Broad research efforts which potentially cut across multiple research domains were identified, including artificial gravity, improved drug delivery and monitoring systems, and the need to optimally integrate inflight and postflight assessment and rehabilitation efforts across disciplines.

National Space Biomedical Research Institute

Workshop on Human Vestibular Adaptation

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November 18-19, 1999

(11/9/99)

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Research Team: *Neurovestibular Adaptation*

Project Name: Context-specific adaptation of gravity-dependent vestibular reflex responses

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Executive Summary

When we move about in the environment, we constantly make use of reflexive motor adjustments in order to maintain posture and balance in reaction to disturbances. Two such motor activities are the movements of the head and the eyes. Impairment of these motor reflexes can lead to disorientation and reduced performance in sensorimotor tasks (such as piloting of spacecraft). Therefore, the adaptive abilities of these systems are important to prevent mishaps during changes in environmental conditions (e.g. gravito-inertial force, gif).

In the absence of a normal earth gravity field, the dynamics of head stabilization, and the interpretation of vestibular signals that sense gravity and linear acceleration, are subject to change. Transitions between different gif environments – as during different phases of space flight – provide an extreme test of the adaptive mechanisms that maintain these reflexes. During extended space flight, crew members may live in artificial gravity and make transitions to weightlessness, planetary exploration, and return to Earth. If they learn sensorimotor skills such as piloting in the normal gravity of Earth, will they be able to perform them adequately in the weightless or the artificial gravity environment? More generally, can people have two different sets of vestibular reflexes, which they are able to switch between rapidly? Are there procedures that could help to transfer (or to inhibit) training from one situation to another? These are the main types of questions addressed by our work. The overall goal of this project is the study of context-specific vestibular and oculomotor reflexes. Special emphasis is placed on the use of gif as a context cue for switching between adapted reflexes. The general approach is to adapt a specific motor response (saccades, VOR, VCR) in one way (e.g. increase in gain) in one gif condition, and another way (e.g. decrease in gain) in another gif condition, and then see if the gif condition itself (the context cue) can recall the previously learned adapted responses.

The knowledge gained from our studies will help us to design adaptation strategies (pre-flight and in-flight) to assist flight crews in making transitions between different gravitoinertial force situations, and can provide design data for spacecraft facilities (artificial gravity, exercise centrifuge) by delineating the limits of human adaptive capabilities.

A prerequisite to the use of context-specific adaptation procedures as a countermeasure is to identify those responses that need to change in a context-specific manner during space flight. There are many physiological changes that occur during flight, but not all of them are adaptive in the sense of bringing performance back to the normal pre-flight level. One must also think in terms of possible detrimental effects during long-duration flight. If reflexes become inappropriately calibrated during extended flight, then this "incorrect" calibration may generalize to the planetary gravity phase. There is certainly evidence that this occurs, as evidenced by difficulties with posture and locomotion immediately after shuttle flight.

Some reflex responses that develop in flight are inappropriate for planetary gravitational fields, while perfectly acceptable for 0g. An example is the putative reinterpretation, in space, of all otolith stimulation as translation (rather than tilt). As there is no true tilt (change in orientation with respect to gravito-inertial vector) in space, the absence of tilt responses is acceptable there. However, when this response configuration generalizes to a planetary gravity environment (whether Earth [1 g] or Mars [0.38 g]), it is inappropriate. Thus we might tailor context-specific adaptation to maintain acceptable planetary responses while in flight, in association with artificial gravity or another stimulus arrangement that simulates one requiring tilt responses.

As an example, two responses which may be amenable to context-specific adaptation follow:

- If artificial gravity is used during long-term flight, it may be desirable to maintain the appropriate sensorimotor calibrations for the rotating (artificial gravity) and non-rotating (weightless or planetary) environments. Normally, exposure to a rotating environment

induces disorientation and inappropriate reflex components, due to the action of cross-coupled (Coriolis) rotational stimuli on the vestibular system. Adaptation would involve training subjects to have the appropriate reflex calibrations while rotating and not rotating, and to switch between the two sets immediately when switching environments.

- Adaptation to the space environment may involve reinterpreting otolith (linear acceleration) stimulation as arising from translation, rather than from combinations of translations and tilts with respect to gravity as on earth (otolith tilt-translation reinterpretation: OTTR). One pre-flight adaptation strategy would be to reduce the tilt component of motor responses to mimic the flight environment (while alternately presenting stimuli to retain the tilt component, to enhance the eventual return to a gravity environment).

Related issues addressed in our experiments include determining 1) effective adaptation schedules, 2) if gravity can be an effective context cue and for which responses, and 3) the role of the cerebellum. The role of the cerebellum in these adaptations is important as a point of fundamental knowledge, but it has serious practical import as well. If, as seems likely, the cerebellum is adversely affected by space flight, then its ability to implement adaptation-based countermeasures is suspect. We would be remiss to propose countermeasures based on adaptation of vestibular responses without assessing the involvement of the cerebellum.

Outline of Individual Sub-Projects

Various experiments investigate the behavioral properties, neurophysiological bases, and anatomical substrate of context-specific learning mechanisms. We use otolith (gravity) signals as the contextual cue for switching between adapted states of the saccadic system, the angular and linear vestibulo-ocular reflexes, and the VCR. (By LVOR we mean the oculomotor response – horizontal, vertical, and torsional – to linear translation of the head and body.)

Context-specific saccade adaptation. We have evidence for context-specificity in human saccades. Two sets of parabolic flight experiments examined the use of instantaneous gravity level (alternating 0 g and 1.8 g) as a context cue for adapted saccadic eye movements. Saccades (rapid eye motions that move the eyes between targets) can be adaptively altered by presenting a target, then moving that target to a new location before the eyes can get to its first location. After several trials, an adaptive sensorimotor mapping takes place, so that the eyes move directly to the new target location when presented with the original target. Ground experiments at Johns Hopkins successfully used vertical eye position, horizontal eye position, head roll tilt, and upright/supine posture as context cues, so that saccades are increased in size in one context (when subjects look upward, or tilt their heads to the right, or are seated upright), and decreased in size in the other context (when subjects look down, or tilt their heads to the left, or are supine). Data from parabolic flight indicate that g-level also can serve as an effective context cue.

Context-specific LVOR adaptation. We demonstrated the ability to use a gravity cue (head orientation) as a context for switching between two different adapted versions of the linear VOR. The gain of the LVOR can be adaptively changed by having the subject view a visual field that moves with him or her on the sled (driving the gain down, since no eye movements in response to head/body translation are required to stabilize the visual field) or view a visual field that moves opposite to sled motion (driving the gain up). We have been able to induce changes in gain that are associated with head roll tilts (context cues) in different directions.

Properties of AVOR and LVOR in squirrel monkey. In the squirrel monkey, we completed baseline investigations of the dynamics of the AVOR with high frequencies and accelerations, revealing interesting nonlinearities which must be understood before adaptive effects can be investigated. Monkey LVOR adaptation studies were also performed, demonstrating adaptive increases and decreases. Torsional eye movement responses to the linear translations did not

change significantly after adaptation, suggesting that the translational and tilt components of the LVOR (horizontal and torsional eye movements, respectively) may not be closely coupled. This has implications for paradigms designed to adaptively change tilt-translation interpretation.

Pursuit and the LVOR in humans and in rhesus money. Results in animals indicate that the LVOR is abolished after flocculectomy, and it is greatly impaired in humans with vestibular deficits as well. Pursuit deficits mirror these changes in the LVOR. This suggests that pursuit and the translational LVOR are tightly linked. A separate set of experiments has demonstrated context-specific adaptation of pursuit gain in humans and monkeys. These two results together may form the basis for a powerful strategy to adapt the otolith-mediated translational LVOR.

Properties and adaptation of head-neck reflexes. Experiments at Baylor College of Medicine on adaptation of the VCR also show evidence of context-specificity. These experiments have established baseline properties of the response along different axes, in terms of mathematical models. Adaptation to an artificial increase in inertia of the head has been demonstrated, as manifest by a decrease in head oscillation during body perturbations. The appropriate adapted response was stored by the head-neck control system even after subsequent re-adaptation back to normal inertia: the system responded appropriately to each inertial load to keep head oscillations at the same level. This capacity to switch between two sets of system parameters persists for at least 35 days after the initial adaptation: the appropriate head damping occurred immediately for both normal and increased inertia loads, showing that two sets of damping parameters can exist simultaneously and be switched in and out as needed.

Adaptation to a rotating environment. Short-radius centrifugation (a form of artificial gravity) is a promising potential countermeasure to long-term weightlessness. Unfortunately, it has a number of side effects related to the unexpected effects of head movements in the rotating environment. Transitions between the artificial gravity (rotating) and weightless (non-rotating) environments will likely cause additional problems. Experiments at MIT are investigating the extent to which these side-effects can be overcome through adaptation. Head movements during centrifugation induce discomfort, non-compensatory vestibulo-ocular reflexes, and illusions of body tilt. Significant adaptation occurred following a series of experimental sessions of head turns during rotation in the light, such that these detrimental effects were reduced.

Key Findings and their Implications

- Saccadic eye movements can be adapted in a context-specific manner, using a number of different context cues. The more relevant the context cue is to the response being adapted, the more effective it seems to be in context-switching (e.g. horizontal eye position is a more effective cue for horizontal saccade adaptation than is vertical eye position).
- The magnitude of gif (during parabolic flight) can be used as a context cue for switching between adapted saccade states. There is evidence for retention of this adaptation after 8 months. The lunar and Martian g levels can recall adaptations imposed during 0 g. (This and the above result satisfy a modified version of aim 1 of the original proposal; the original aim involved adaptation of the AVOR, but saccade adaptation is more easily accomplished in parabolic flight, and there is evidence that saccade accuracy may be adversely affected during flight, due to alterations in static torsional eye position. The essential component of the aim – use of g level as a context cue – was achieved.)
- Compensatory eye movements made in response to translational (LVOR) can also be made context-specific, using the orientation of gravity with respect to the head (head tilt) as a context cue. For inter-aural translations, head roll is a more effective context cue than is head pitch. This is analogous to the situation with saccade adaptation: the closer the context cue is to the response being adapted, the more effective it is. (This satisfies a modified aim

2 of the proposal. It was proposed to adapt phase rather than gain, but gain adaptation has turned out to be easily accomplished, and has more countermeasure relevance.)

- Sensorimotor adaptation to head movements during short-radius centrifugation (23 rpm, 1 g at the feet) occurs, as quantified by measures of inappropriate vertical eye movements, motion sickness, and illusory tilt. Three ten-minute adaptation sessions produced adaptation that was retained (at reduced level) a week later. Adaptation to head movements to one side did not generalize to head movements in other directions. Full adaptation did not take place; while motion sickness disappears after 10 adaptation sessions, vertical nystagmus and illusory tilt do not. Context-specificity of the adaptation is apparent since subjects did not experience motion illusions when off the centrifuge between test sessions. (This satisfies aim 3 of the original proposal. Subjects can acquire adaptation to short-radius centrifugation, and move between rotating and normal environments without detriment.)
- Properties of the head-neck control system (VCR) in three dimensions (roll, pitch, yaw) can be adequately modeled by a relatively simple, 2nd-order linear system, plus a single dead-zone nonlinearity. Adaptation of this system to changes in head inertia can be induced. This adaptation can be made dual-state, such that the appropriate neural control mechanisms for head stabilization change modes immediately upon a change in head inertia. (This satisfies aims 4 and 5: modeling of the vestibular contribution to head stabilization has been accomplished, short-term adaptation has been demonstrated, and some measure of context-specific adaptation to immediate and repeated changes in head inertia has been shown.)
- Bilateral removal of the flocculus and paraflocculus in rhesus monkey produced almost complete loss of the horizontal LVOR (even after the angular VOR had recovered). Likewise, human cerebellar patients have comparable defects in pursuit and the LVOR, while the AVOR appears to be controlled independently. This suggests that the vestibulocerebellum plays a critical role in the generation of the LVOR, and that there is a tight relationship between the generation of the LVOR and smooth pursuit. This has implications for countermeasures that are based on adapting translation versus tilt responses mediated by the otoliths. (This satisfies multiple aspects of aim 6: the role of the vestibulocerebellum in the LVOR, and the role of pursuit in the generation of the LVOR.)
- A separate experiment showed systematic variations in the axis of eye rotation at different vertical elevations, during pursuit, AVOR, and LVOR. Axis tilts for pursuit and LVOR were almost identical, and different from that for the AVOR, again showing a close relationship between neural processing for pursuit and the LVOR. (This satisfies the remaining portion of aim 6: assessment of axis of rotation in pursuit, LVOR, and AVOR.)
- Context-specific adaptation of smooth pursuit eye movements has been demonstrated in both humans and rhesus monkeys. Using vertical eye position as a context cue, the initial acceleration of the eyes, when presented with a moving target, can be made to decrease with the eyes elevated, and to increase with the eyes depressed. This has implications for context-specific adaptation of some types of otolith-mediated responses, which seem to be at least partly expressed through the pursuit system (see above). (This partially addresses aim 8, which was intended to determine the role of the vestibulocerebellum in context-specific LVOR adaptation. Although the original aim was not dealt with directly, progress was made in the general area by determining the role of the cerebellum in pursuit and the LVOR, and by demonstrating context-specific adaptation of pursuit.)
- LVOR gain adaptation was induced in squirrel monkeys, and was specific to the frequency used for adaptation. Following adaptation of LVOR gain, there was no significant change in

the torsional eye movements to head tilt, suggesting that the responses to head tilt and head translation are not tightly coupled. (This is the initial stage of aim 7, meant to determine the role of the vestibulocerebellum in the adaptive control of the gain and phase of the LVOR. Other parts of aim 7 are still under investigation, and have had to await the development of equipment and procedures for LVOR adaptation in the monkey, and lesioning of same.)

As pointed out above, although much of aim 8 (role of the vestibulocerebellum in context-specific LVOR adaptation) was not addressed directly, a number of related experiments in humans (not all of which were originally proposed) have made a significant contribution to the overall goal of this aim. In particular, elucidation of the role of the cerebellum in pursuit and the LVOR, demonstration of the close connection between these two responses, and the production of context-specific adaptation of both the LVOR and pursuit, all contribute to understanding the role of the cerebellum in these adaptive processes. This made some of the specific proposed monkey experiments relatively less important. The animal work continues to have relevance, however, in that it will allow more extensive testing over a range of stimulus parameters, and localization of those cerebellar pathways which contribute to adaptation.

Additional Implications, Relationship to NASA Critical Path Issues

Neurovestibular problems have been identified and listed on the NASA "Critical Path Roadmap" for serious problems that could affect a mission to Mars. Some indication of the range of problems and their severity is found in the May 1997 "Final Report of the NASA Task Force on Countermeasures," which states: "Based on the experience of both the cosmonauts and the astronauts, it is apparent that the ability to egress suddenly will be limited unless effective countermeasures for the loss of neuromuscular performance are identified and adhered to rigidly during prolonged spaceflights" (p. 9). Specific problems listed in the report include changes in eye-head coordination, decrements in postural control, sensory illusions such as otolith tilt-translation reinterpretation, and "flashbacks" between 1g and 0g states with associated motor dysfunction. Concerns were raised for the effects of these problems on vehicle control and unassisted egress. The issue of "flashbacks" is especially interesting relative to our work, as it indicates the simultaneous existence of two adapted states (one for 0g and another for 1g). Knowledge about how to avoid such inadvertent flashbacks, as well as how to make use of contextually-gated dual-state adaptation, is the central aim of all of our studies in this project.

An especially useful aspect of our parabolic flight experiments is that we fly in consecutive years. With the same subject tested each year, we can assess how much the 0 g responses have been maintained throughout the intervening period of 1g exposure. This is particularly germane to Mars missions, when gravity-based responses which may have been trained before flight may have to be recalled in the Martian gravity environment many months later.

Not only do our studies provide valuable information for the development of countermeasures, they will also provide basic information on adaptive neurovestibular processes. This is especially true of experiments dealing with the role of the cerebellum in motor control, and signal processing of otolith information for the generation of reflex responses in different environments.

One specific clinical implication of these studies is in the area of vestibular rehabilitation (and physical rehabilitation in general). Rehabilitation exercises are generally learned and carried out under supervision in a clinical setting. There is the possibility that inadvertent contextual cues in this setting will be associated with improved performance while in the clinical setting, which will not transfer completely to settings of normal daily living. In this respect, it is useful to know what context cues are most effective, what types of responses can be made context-specific, and how to avoid such context-specificity when it is detrimental (i.e. when generalization is desired).

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I. Project Research Activity

Aims 1 & 2: Saccade and LVOR Adaptation

To make the results from these two aims easier to follow and place into context, the following list outlines background experiments, and previous and ongoing NSBRI experiments (labeled as such), that pertain to these aims:

- **Saccade** gain (horizontal), **horizontal eye position** context cue (NSBRI)
- **Saccade** gain (horizontal), **vertical eye position** context cue (NSBRI)
- **Saccade** gain (horizontal), head **roll** context cue (NSBRI)
- **Saccade** gain (horizontal), **upright vs. supine** context cue (NSBRI)
- **Saccade** gain (vertical), **horizontal eye position** context cue (NSBRI)
- **Saccade** gain (vertical), **vertical eye position** context cue (NSBRI)
- **Saccade** gain (horizontal), **gravity** magnitude context cue (NSBRI)
- AVOR gain with vertical eye position as context cue (prior published work)
- AVOR, gain adaptation, vergence angle context cue (prior published work)
- AVOR, gain adaptation, head orientation context cue (prior published work)
- AVOR, 0.2 Hz, phase adaptation, no context cue (prior published work)
- AVOR, 0.2 Hz, phase adaptation, type of eye movement context (prior published work)
- **LVOR**, 0.5 Hz, gain and phase adaptation, 20 min, ×1, no context cue (NSBRI: submitted)
- **LVOR**, 0.5 Hz, phase adaptation, 20 min, 45 deg lead or lag, no context cue (NSBRI: in press)
- **LVOR**, 0.5 Hz, phase adaptation, 30 min, 45 deg lead/lag, **vertical eye position** context cue (NSBRI)
- **LVOR**, 0.7 Hz, gain adaptation, 1 hr, ×0 head upright, test upright and rolled 45 deg (NSBRI)
- **LVOR**, 0.7 Hz, gain adaptation, 1 hr, alternate ×0 head upright and ×1 head **roll** 45 deg (NSBRI)
- **LVOR**, 0.7 Hz, gain adaptation, 1 hr, alternate ×0 head upright and ×1 head **pitch** 26 deg (NSBRI)
- **LVOR**, 0.7 Hz, gain adaptation, 1 hr, alternate ×0 head up and ×2 head down **pitch** 26 deg (NSBRI)
- **LVOR**, 0.7 Hz, gain adaptation, 1 hr, alternate ×0 head left and ×2 head right **roll** 45 deg (NSBRI)
- **LVOR**, 0.7 Hz, gain adaptation, 1 hr, alternate ×0 head left and ×2 head right **roll** 26 deg (NSBRI)

Aim 1: Context-specific Adaptation in Parabolic Flight

PI: Shelhamer. Assisted by D Roberts, R Clendaniel, T Roberts, and the NASA Reduced Gravity Program Office and Human Test Subject Facility (parabolic flights).

Introduction

Saccades are rapid reorienting movements of the eyes that change the line of sight. They represent an important sensorimotor behavior that is required during such activities as spacecraft piloting and egress, and can serve as a model for more complex behavior. Saccades are, to a large extent, ballistic. That is, when a neural decision is made to generate a saccade, that saccade will begin about 200 msec later, despite any new visual information that would alter the original saccade. Because of this, if a visual target is moved while a saccade to that target is under way, the saccade will go to the original target location and a second saccade must be made to the new

target location. Eventually, saccades are made directly to the second target position, effectively changing the gain of the system (i.e. the amount that the eye moves as a function of the observed target position). This property is useful as a paradigm for adaptation of the saccadic system. Saccadic adaptation has been studied extensively and is an ideal test bed for models of adaptation.

Our goal in this set of experiments is to increase adaptively the gain (amplitude) of saccadic eye movements in one context, adaptively decrease the gain in another context, and then see if the context itself can recall the associated saccade gain. This is the only one of our experiments directly to make use of the magnitude of gif (in parabolic flight) as a context cue, which is the closest that we can come to recreating the g variations of the space environment.

Procedures – ground experiments

In preparation for our parabolic flight experiments, we performed a series of ground-based experiments on context-specific adaptation of saccadic eye movements. Adaptation is carried out with a standard double-step paradigm, as follows. In a dark room, an LED target 10 deg to one side is illuminated. When it is extinguished, another target, 5 deg to the other side (i.e. 15 deg from the first target) is lit. As the subject makes a saccade to the new target, it is extinguished (when the eyes are approximately half way to the target), and a final target at 10 deg (20 deg from the first target) appears. A corrective saccade is made to this final target. As adaptation progresses, the subject learns to make a 20 deg saccade, directly to the final position, when presented with a target that is 15 deg away from the present eye position. This process is repeated for saccades in both directions, to the right and to the left, and results in a gain increase (15 deg target displacement results in 20 deg saccades). A similar paradigm can be designed to ask for 10 deg saccades when presented with a 15 deg displacement; this is a gain decrease paradigm.

In the first set of experiments, we used horizontal eye position as a context cue for horizontal saccades. A gain increase paradigm was presented for 19 trials with all targets to the right of the subject's midline, and a gain decrease paradigm was presented for 19 trials with all targets to the left of the midline. These two alternating conditions were repeated 19 times, for a total of $38 \times 19 = 722$ trials. A similar set of trials was performed using vertical eye position as a context cue, again for horizontal saccades: a gain increase paradigm was presented for 19 trials as subjects looked up 10 deg, and a gain decrease paradigm was presented for 19 trials as subjects looked down 10 deg. These two conditions were alternated as above.

The next set of experiments used head orientation with respect to gravity as a context cue (for horizontal saccades). With the head tilted (rolled) 45 deg to the right, a gain increase was asked for, and with the head tilted 45 deg to the left, a gain decrease was asked for. Saccades were always horizontal with respect to the subject, so that eye position was not an inadvertent context cue as the head changed position. Increase and decrease conditions were alternated as described previously.

We next performed a set of experiments using upright and supine positions of the subject as the context cue (for horizontal saccades). A head-mounted visual display, built in our laboratory, was used to present LED targets against a black background. This display moves with the subject and is essential for this experiment and those carried out in parabolic flight. (We considered the use of a virtual reality device for this experiment, but latency considerations quickly ruled out this possibility. These displays update the entire image every 33 msec, while saccade durations are on the order of 50 msec. If a saccade begins immediately after the image has been refreshed, and the eyes do not reach the halfway point until the next image, then 99 msec will have elapsed until the target position can be changed. This is clearly too long, and so a very simple head-mounted unit was designed, using LEDs under computer control.) Target locations in this display were not the

same as those in the testing room used for the previous experiments, thus gain-increase adaptation asked for a change in saccade size from 22 deg to 28 deg, and gain-decrease adaptation required saccades to change from 22 deg to 16 deg.

The final ground-based experiments tested adaptation of vertical saccades, using vertical and horizontal eye position as context cues. The paradigms are as described above, except that vertical saccades are adapted. This allows us to determine if the effectiveness of eye position as a context cue is related to the direction (horizontal or vertical) of the saccades being adapted.

In each of these experiments, a set of test trials was presented before and after adaptation. These trials are identical to the double-steps used for adaptation, except that the second target does not appear; there is no feedback to the subject as to the accuracy of the primary saccade, and no drive for adaptation. This provides an open-loop test of the saccade visuo-motor programming, and is a standard procedure in all of our saccade adaptation experiments.

Procedures – parabolic flight

Adaptation of horizontal saccades was carried out on board NASA's KC-135 aircraft during parabolic flight, for two weeks in 1999 (year 1 flights) and two weeks in 2000 (year 2 flights). Flying in a parabolic trajectory, the aircraft produces alternating periods of approximately 0 g and 1.8 g, for approximately 25 sec each. This sequence is maintained for several cycles, ranging from 3 to 20, until operational considerations, weather, turbulence, or airspace limitations require the aircraft to turn, whereupon the parabolic trajectory is started again. A typical flight consists of 40 parabolas, lasting about 2 hours.

Three subjects performed the experiment in flight in year 1, two of them for several consecutive days. In year 2, four subjects performed the experiment, one of the them being a repeat subject from year 1. Experimental conditions during flight are not perfectly predictable: timing of the parabolas may vary as noted, and gravity levels are subject to some variation. For these reasons, it is not possible to organize an experimental session as on the ground; thus, test trials were interspersed at various times during the flight rather than being placed only at the beginning and the end.

In the second year of flights, the following subsidiary goals were addressed in addition to the primary goal of demonstrating context-specific saccade adaptation per se: 1) retention of adaptation in one subject from year 1 to year 2 (eight months between flight sessions); 2) transfer of context-specific adaptation from upright to supine orientation; 3) whether or not the supine orientation could serve as an analog to the 0 g phase of flight, in terms of recalling adaptation induced during the 0 g phase of flight; 4) transfer of adaptation induced in the 0 g phase to lunar (0.6 g) and Martian (0.3 g) g levels; and 5) the effects of different adaptation schedules (presentation of only one context at a time for several parabolas rather than juxtaposing the two contexts with each parabola).

Unfortunately, not all of these subsidiary experiments yield conclusive results, due to the small number of trials that were available to test each one (without sacrificing the main objective of demonstrating context-specificity based on g level). This loss is offset, however, by the fact that we were able to run two subjects simultaneously (with minimal changes in equipment) during the second week of our year 2 flights, due to software and hardware changes made on-site by the PI and engineer Dale Roberts, and due to the cooperation of NASA personnel in making space for an extra subject available on board the aircraft.

Another change from the ground experiments was the use of EOG to measure eye movements (EOG was also used in the upright/supine context experiments described above), as the use of a search coil system in the aircraft would be prohibitive. To accommodate the increased noise level and baseline drift prevalent with EOG, the paradigm used a windowing technique to drive the

stimulus based on eye position. When the eyes were fixed on the initial target with little variation, the trial began. The target jumped, and when the eyes were sufficiently far from the initial location, the target made its second jump. Thus, relative and not absolute eye positions were used to trigger target motions, which allowed for EOG signal drift. Since the eyes began and ended each trial at known positions, these initial and final positions were used to provide a trial-by-trial calibration of the eye movement recordings. This was necessary because of the artifacts and drift associated with EOG, especially in an environment such as parabolic flight.

Adaptation trials in parabolic flight (and the upright/supine series) had different amplitudes than those in the majority of the other studies; they are described above in the upright/supine section. The head-mounted visual display described above was used for these experiments.

Results – ground experiments

In all experiments, a consistent set of criteria was used to determine the size of the initial (primary) saccade in each trial. Subsequent saccades were considered as part of the primary saccade if they occurred within 200 msec of one another.

The first sets of experiments used horizontal and vertical eye position as context cues. Using horizontal eye position, in two subjects we found consistent and reliable context-specific adaptation. With the eyes to the right, gain was increased, and with the eyes to the left, gain was decreased. Vertical eye position was somewhat less effective as a context. Gain decrease with the eyes downward was significant, while gain was less affected (and not significantly so) with the eyes upward (asking for a gain increase). It is well established that gain decrease adaptation for saccades is more easily accomplished than gain increase adaptation; our result confirms this and suggests that the adapted responses are not purely volitional, as we would then expect changes to be significant for gain increase as well as decrease.

Positive results were found using head orientation (roll tilt) as a context cue, as shown in Fig. 1.1 below. The × symbols represent the size of the primary saccade in each gain-increase trial; the **o** symbols are the size of each primary saccade in the gain-decrease trials. Saccades begin with an amplitude of almost exactly 15 deg, which is the displacement of the initial target. With adaptation, the size of the saccades decreases or increases, as appropriate for the adaptation condition. Perfect adaptation would have the ×'s along the 20 deg line, and the **o**'s along the 10 deg line. As above, gain decrease was more readily accomplished than was gain increase.

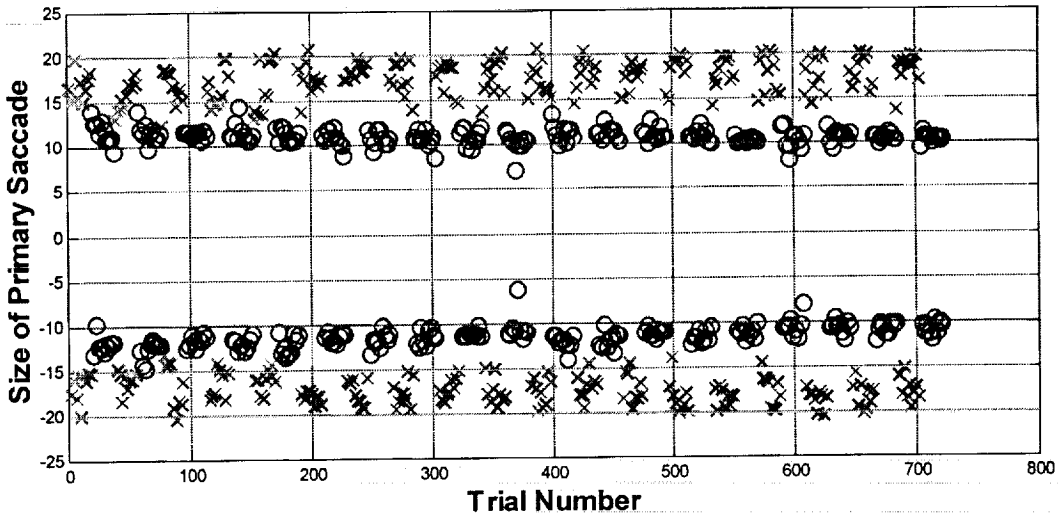


Figure 1.1. Context-specific adaptation of saccade gain, using head roll tilt as a context cue. × = size of primary saccade for gain-increase trials; **o** = size of primary saccade for gain-decrease trials.

The final set of ground experiments on horizontal saccade adaptation, using upright and supine orientations as contexts, was also successful. All of these experiments are summarized in the graph in Fig. 1.2. Each pair of bars represents a single context-specific experiment, with the patterned bar showing the change in saccade size (difference between post-adaptation and pre-adaptation test trials) under gain-decrease adaptation, and the empty bar showing the change under gain-increase adaptation. (The results labeled "control" are explained below.) In each case, note that the gain-decrease adaptation is substantial, while the gain-increase adaptation is usually more meager, and sometimes in the incorrect direction (as in the first two cases with vertical eye position as a context). The use of upright and supine orientations produces some of the best results.

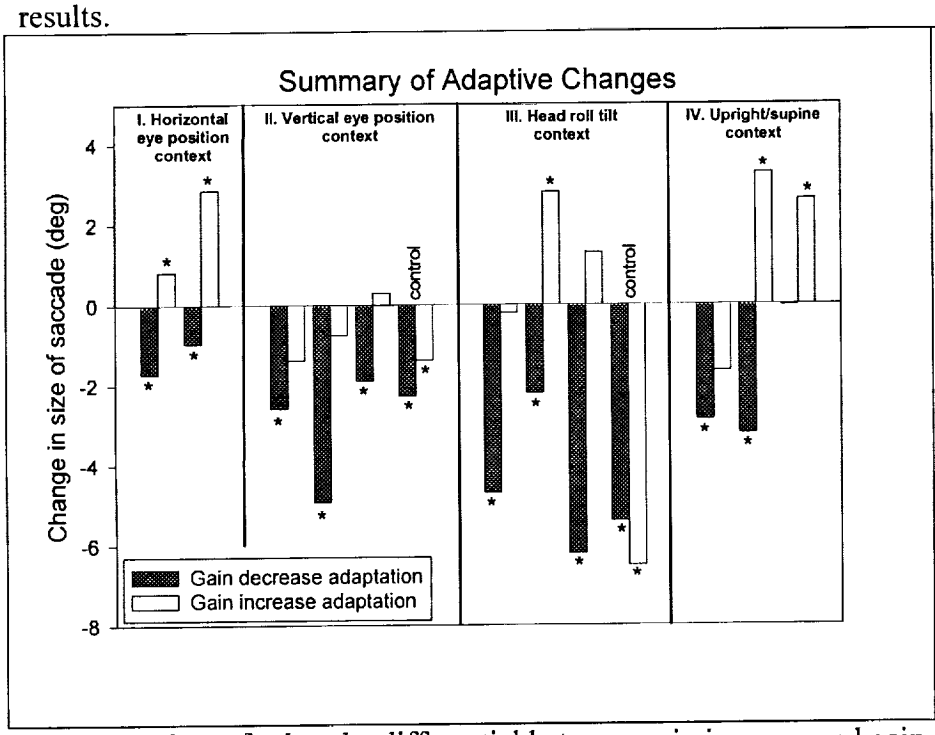


Figure 1.2. Summary of ground-based experiments on context-specific adaptation of saccade gain. Each pair of bars represents a single experimental session, in which saccade gain was adapted upward in one context and downward in another context, with the contexts alternating over the course of the session. To the far left are sessions using vertical eye position as a context cue. In the center are sessions using head roll (to the right and to the left) as a context. To the right, upright and supine postures were used as a context. See text for a full discussion.

To investigate further the differential between gain increase and gain decrease in the experiments above, two control experiments were performed. In one case, we repeated the context experiment described above that used vertical eye position as the context cue, with one modification: during the portions of the adaptation paradigm when the eyes were up, when gain-increase adaptation would normally be called for, the target lights were covered and no adaptation was called for (sham trials); the subject made saccades at will. Gain-decrease adaptation trials, with the eyes lowered, occurred as in the original experiment. In this control experiment, after adaptation, the gain was decreased with the subject looking down, as expected, and the gain was decreased also with the subject looking up. That is, the adaptation had generalized from the situation with the eyes down to the situation with the eyes up – it had transferred from one context to the other.

In Fig. 1.3 below, the left panel shows trials from the original experiment using vertical eye position as a context, and the right panel shows trials from the control experiment just described. Test trials are shown (as described earlier: after the primary saccade had begun, the second target light did not appear; there is no corrective saccade to the second target, and this measure of open-loop performance is an indicator of the extent of adaptation). The ×'s represent trials with the eyes raised 10 deg (gain increase trials on the left, sham trials on the right); the **o**'s represent

trials with the eyes down 10 deg (gain decrease). In the control experiment, there is transfer of adaptation from the context used during adaptation (eyes down) to the eyes-up context which experienced no adaptation. The context experiment shows that although gain-increase adaptation is not as strong as gain-decrease adaptation, exposure to the gain increase condition with the eyes up does prevent generalization of the gain-decrease adaptation. Thus, in the context experiments above, although exposure to the gain increase paradigm with the eyes up may not confer a large amount of adaptation, such exposure does serve to prevent the transfer of adaptation from one context to another.

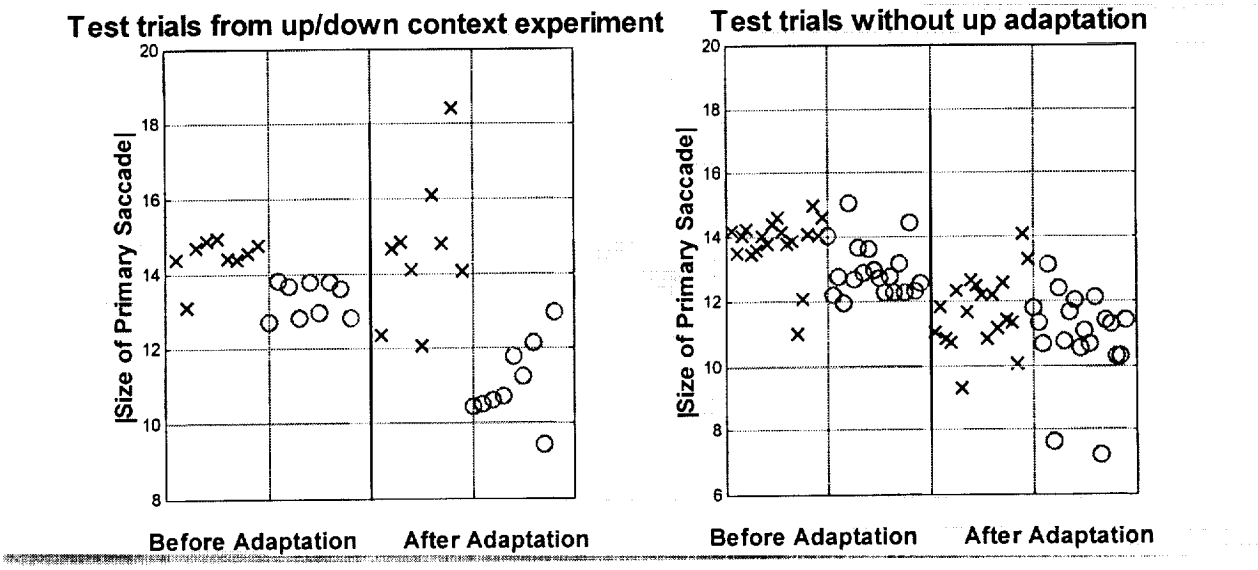


Figure 1.3. Context-specific adaptation of saccade gain, using vertical eye position as a context cue (left) and a similar experiment which contained no adaptation trials during what would otherwise be the eyes-up gain-increase trials (right). × = size of primary saccade for gain-increase trials (left) or sham trials (right); o = size of primary saccade for gain-decrease trials. Test trials are shown, in which a second target does not reappear after the first is extinguished (see text).

A similar control experiment was performed for the use of head tilt as a context, with similar results: using with sham adaptation trials with the head tilted to the right, and gain-decrease adaptation trials with the head tilted to the left, the decreased gain generalized to both head orientations. These control experiments are shown in Fig. 1.2, labeled as "control". Note that in each case, the gain in both contexts (both bars in each pair) has decreased, more so than in the other (non-control) experiments.

Adaptation of vertical saccades, using vertical eye position and horizontal eye position as context cues, was also generally effective. Further analysis will attempt to determine which context cues are most effective for each saccade direction.

Results – parabolic flight experiments: behavior during adaptation trials

The primary goal of the parabolic flight experiments was to determine if instantaneous g-level (more appropriately, gif level) can be used as a context cue. An example from one subject on one flight is shown in Fig. 1.4. As noted previously, it was not always possible to carry out the sequence of test and adaptation trials according to our desired plan. Thus, we present in this section data from adaptation trials only. (This analysis has not been completed on all subjects and all flights.) Shown in the graph is the first trial upon each change in context (gravity level). That is, each time the g-level changed, the adaptation trials switched from gain-increase to gain-

decrease (or vice versa). The very first trial upon such change indicates whether or not the saccade adaptation has truly become associated with the instantaneous g-level. As shown in the graph, adaptation is indeed context-specific. As in the ground experiments, gain-decrease adaptation is stronger than gain-increase adaptation.

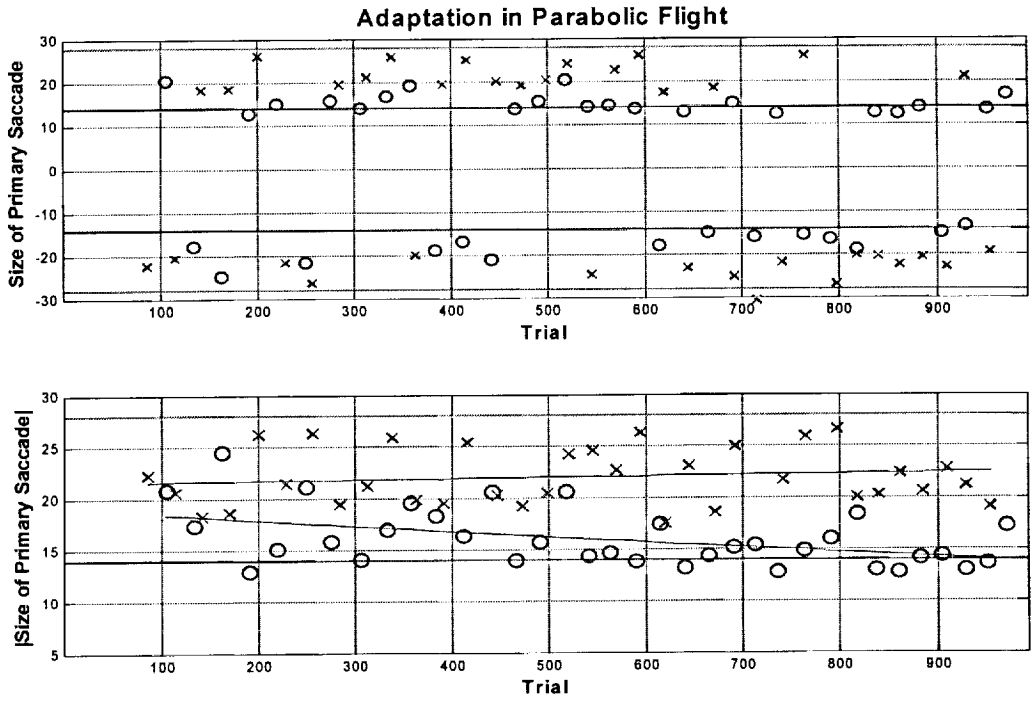


Figure 1.4. Example of context-specific saccade adaptation in parabolic flight. × = size of primary saccade for gain-increase; o = size of primary saccade for gain-decrease trials. Each symbol represents the first trial in each set of contiguous adaptation trials for a given gravity state (see text). The top graph shows saccades to the right as positive and to the left as negative, while the bottom graph shows the absolute value of each saccade. Initial veridical saccade amplitude was 21 deg; gain decrease-adaptation had a desired amplitude of 14 deg, and gain-increase adaptation a desired amplitude of 28 deg (reference lines are shown in each graph at these amplitudes). In the bottom graph, regression lines are shown, indicating the time course of adaptation. Gain-decrease adaptation was more complete than gain-increase adaptation, as noted by the different slopes of the two regression lines.

Results – parabolic flight experiments: behavior during test trials

A more complete discussion of the results of the flight experiments follows. Each subject is taken in turn. The associated graphs can be found in Appendix A. These data are presented here at some length because significant effort and expense were devoted to the parabolic flights, this experiment is one of the cornerstones of our project since it is the only one to deal with gravity level per se as a context cue, and each subject was exposed to a slightly different schedule of adaptation trials in flight.

Subject C performed the experiment only one day, with test trials in 1 g flight before the parabolas, and test trials while upright and supine at the end of the flight. It is apparent from the pertinent graph in Appendix A that the adaptation develops over the course of the flight, as seen by the increasing difference between saccade sizes for the two directions of adaptation (increase and decrease). None of these differences is significant, however (at the 0.10 level). There is a significant difference ($p<0.0001$) between increase trials while upright, and decrease trials while supine, during 1 g flight after adaptation. The gain decrease saccades are also significantly smaller while supine after adaptation than in 1 g flight (upright) before adaptation ($p=0.0011$),

while gain increase saccades are not significantly different. These results suggest that the supine orientation in 1 g may serve as a surrogate for 0 g after context-specific adaptation to alternating 0 g and 1.8 g.

Subject B1 performed the experiment in year 1 for three consecutive days. Comparison of gain-decrease and gain-increase saccades during each session of test trials yields only two cases in which they are significantly different (at $p=0.10$): during parabolas at the start of flight 3 ($p=0.06$), and during parabolas at the end of flight 3 ($p<0.0001$). Thus adaptation seems to have accumulated over the course of the three flights, even to the extent that it was significant at the start of the third day's flights, before any adaptation exposure on that day. The fact that there was not a significant difference at the end of flight 2, while there was a difference at the start of flight 3, suggests a form of consolidation, in which the adaptation from flight 2 was solidified during the intervening non-flight period (akin to the fact that many fliers report motion sickness at the end of a flight, only to feel much better during the entirety of the subsequent flight). This consolidation or solidification is manifest most obviously as a decrease in the variability of the sizes of the saccades from the end of flight 2 to the start of flight 3. Another indication of the adaptation can be found by comparing test trials at the end of flight 3 and to those during the first parabolas of flight 1; while gain-decrease trials have not changed, gain-increase trials have ($p<0.0001$). Likewise for a comparison of trials at the end of flight 3 with those in 1 g at the start of flight 1 ($p=0.0006$ for gain-increase). That this effect is not merely an adaptation to the context of the experimental apparatus and environment from flight to flight can be seen by comparing test trials in 1 g flight from flight 2 to flight 1 and from flight 3 to flight 1; in neither case are there significant differences (comparing gain-decrease saccades across flights, and gain-increase saccades across flights). Unlike subject C, there was no significant difference between gain-increase saccades while upright, and gain-decrease saccades while supine, in 1 g at the end of flight 3.

Subject A has many cases in which gain-decrease and gain-increase trials within the same group are significantly different, including 1 g trials at the start of flight 1 ($p=0.09$; see below for why this might be so) and parabolas at the start of flight 2 ($p=0.02$). The other cases are interesting as they demonstrate a steadily-increasing acquisition of context-specific adaptation. There is no significant difference between increase and decrease saccades at the start of parabolas on flight 1, but there is by the end of the flight ($p=0.00005$). Most notably, on flight 3, when several sessions of test trials occurred, there is a steady increase in the difference between increase and decrease saccades, and a steady increase in the statistical difference between them as well (for test sessions 2 through 7 in flight 3: $p=0.000002$, 0.03, 0.007, 0.0018, 0.097, 0.0003). This increased effect is apparent up to the final two test sessions on that flight (note that there were no useable gain-increase test trials in test session 8). On flight 4, the start of the flight shows the largest adaptation effect seen in this subject, possibly an effect of consolidation of adaptation during the intervening hours after flight 3. Differences between increase and decrease saccades are significant during test sessions at the end and the beginning of flight 4 ($p=0.0005$, 0.0000007). (Due to aircraft problems, flight 2 was cut short by a few parabolas, a day was skipped, and flights 3 and 4 occurred the morning and the afternoon of the following day). The results from flight 3 in particular demonstrate a striking display of the steady acquisition of adaptation over the course of a flight. The dramatic adaptation apparent at the start of flight 4 may in fact be due to consolidation during the several hours after flight 3, and if so is remarkable as it demonstrates that consolidation may be more effective than are subsequent adaptation trials during flight 4.

Subject D flew for 4 consecutive days in year 2. Adaptation was presented on a modified schedule, with one half of each flight devoted to only one type of adaptation (increase or decrease), and the other half to the other type. Comparison of increase and decrease test trials produced no significant differences except for flights 1 (although in the wrong direction) and 2 in 1 g flight ($p=0.03$, 0.09), parabolas at the start of flight 2 ($p=0.017$), and at the end of flight 4 ($p=0.046$). This last result indicates that adaptation has developed over the course of the flights. However, in marked contrast to subject A, there is no steady acquisition of adaptation over the course of the testing sessions, either between flights or within a single flight. This might be attributed to the modified adaptation schedule used for subject D. Note that at the end of flight 3, test trials were performed during parabolas while the subject was supine, to look for transfer of context-specific adaptation between the upright and supine orientations. Due to the lack of significant adaptation at that time even while upright, this test was inconclusive.

Subject B2 flew for 2 days in year 2 (this is also subject B1 in year 1). The adaptation trials were scheduled so that they came in segments of approximately 5 parabolas each: for 5 parabolas gain-increase adaptation trials were presented during 1.8 g with no trials during 0 g, and for the next 5 parabolas gain-decrease trials were presented during 0 g with no trials during 1.8 g; this was repeated throughout the flight, interrupted by test trials. Comparison of increase and decrease trials produced all significant differences. That is, all paired bars in the pertinent graph are significantly different from each other. (Again, see the below for why this might be so for the 1 g trials on the first flight.) Rather remarkable is the fact that there is significant adaptation manifest during the very first parabolas ($p=0.0016$). Compare this to the lack of any such effect during this subject's first flight the previous year (graph for subject B1 in Appendix A). There appears to be significant retention of adaptation from the end of the flights in year 1 to the start of flights in year 2, a gap of 8 months. Also of note is the comparison of the test trials performed under lunar and Martian gravity conditions (approximated during flight by parabolas flown at 0.2 ± 0.05 g and 0.4 ± 0.05 g respectively). Test trials in these cases were compared to those made in 1.8 g during the surrounding parabolas. In both lunar and Martian g fields, saccade sizes are significantly different from those in the surrounding 1.8 g fields, indicating that these two planetary g levels are sufficiently low to recall the saccade gain state that was adaptively imposed during 0 g. In both flights, saccades made in the smaller lunar g field are smaller than those in the relatively greater Martian g field, which suggests that the 0 g-adapted state is recalled most strongly in a 0 g field, and less so as the g field becomes larger.

Subject E flew for two consecutive days. As for subject B2, adaptation was presented in segments of 5 parabolas. Unfortunately, this subject produced only a single significant comparison between increase and decrease saccades: trials in lunar gravity at the end of flight 1 were significantly reduced in size relative to those made in the surrounding 1.8 g portions of flight. This again demonstrates that lunar gravity can effectively recall adaptation imposed in 0 g. Perhaps even more so, these specific results point up the difficulty of experimentation in parabolic flight, as the lack of significant findings can be attributed largely to the small number of trials obtained with this subject (a total of 127 test trials during parabolas, versus 297 for subject B2, who flew the same number of flights with approximately the same scheduling of trials). Note in particular the missing bar for lunar trials in flight 2, and the lack of error bars in some places where only one trial was obtained for each bar.

Subject F flew for 4 consecutive days. During the entire first flight, only gain increase adaptation trials were presented, during the 1.8 g periods, with no trials presented during the 0 g periods. During flight 2, only gain-decrease adaptation trials were presented, during 0 g periods. These two adaptation schedules were repeated for flights 3 and 4. This subject's data is

unfortunately contaminated by high noise level and other artifacts, preventing clear conclusions from being made. Nevertheless, it is clear that during the first flight, gain-increase adaptation generalizes from the 1.8 g to the 0 g phases of flight. This generalization is apparent on the second and third flights as well. The only clear conclusion from this subject's data is that, if adaptation is not presented in both g-level contexts, it will generalize across g levels. This is analogous to the situation described above in the ground experiments.

Discussion of parabolic flight experiments

Results are somewhat subject-dependent, which is not surprising in an experimental environment such as parabolic flight. Nevertheless, the overall evidence indicates that instantaneous gif can serve as an effective cue.

Retention of adaptation (subject B1/B2) is perhaps not surprising; the experiment operators themselves had a significant reduction in motion sickness symptoms on their first flight in year 2 as opposed to year 1, and astronauts report greatly reduced motion sickness during their second and subsequent flights relative to their first, even with many months or years in between.

An issue which arose in some of the data above requires some explanation. In some cases (subject A start of flight 1, B2 start of flights 1 and 2), before any adaptation exposure, saccades from gain-decrease test trials are smaller than those from gain-increase test trials. In most cases this is not a significant effect, and when it is, subsequent adaptation trials increase the difference between the decrease and increase saccades. We believe the following explains this effect. In order to obtain enough test trials during flight, the time between test trials was set at a relatively brief 0.8 sec. This is short enough that, after a few trials, there may be some anticipation of the pattern of target presentation (which is different for gain-increase and gain-decrease trials) for a given type of test trial. In addition, at the close viewing distance used in flight, each target was approximately 1.5 deg in diameter. A subject could thus choose to look at the left edge or the right edge of each LED and still be "on target," allowing about 1.5 deg of leeway short of any adaptation.

In some of the flights, a modified schedule of adaptation trials was used. In an attempt to reduce interference between contexts, adaptations of only one type were given in one g level, with no adaptation presented during the other g level. Then the other adaptation type was presented during the other g level, sitting out the opposite one. This was carried out for anywhere from 5 to 40 parabolas with different subjects. This type of consolidation-oriented scheduling of adaptation trials is not effective. Presentation of increase trials alone in 1 g while sitting out the 0 g phases without adapting, for example (see flight 1 subject F) produces an increase in all saccades, even those tested in 0 g. In the following flight, all decrease trials were presented, which then had to compete with the previous flight's increases in gain. This back and forth battle seems to be more detrimental than beneficial to the acquisition of context-specific adaptation. What does seem to be effective is waiting several hours after adapting before being tested again (as for subject A). This is undoubtedly effective because the contexts used in this experiment – 0 g and 1.8 g – are not likely to be experienced by subjects in the intervening time between successive flights.

Aim 2: Context-specific Adaptation of the Human LVOR

PI: Shelhamer, Zee. Assisted by G Peng, P Trillenberg, S Hegemann, V Patel.

Introduction

During lateral sinusoidal motion, the otolith organs of the vestibular system transduce linear acceleration, and drive the eyes so as to compensate for this motion. The resulting eye movement is known as the linear vestibulo-ocular reflex (LVOR). The LVOR at low frequencies (below

approximately 0.5 Hz) is quite variable and seems to be closely allied with the ocular smooth pursuit system. At higher frequencies, the response becomes more robust and reflexive. The adaptive properties of the LVOR – the ability to change the amount of eye movement made for a given amount of head movement – have only recently begun to be explored. Our current study, described here, is aimed at inducing context-specific adaptation of the LVOR, so that the gain (eye movement / head movement) is increased in one context (head orientation) and decreased in another context (different head orientation), with the context cue able to switch immediately between the two gains.

Elucidation of these adaptive properties of the LVOR is important in the design of space flight countermeasures because, in the absence of the usual gravity vector, the neural processing of otolith signals (which transduce linear acceleration, of which gravity is one type) presumably changes. Thus potential countermeasures might be based on these results. On more general terms, it would be useful to know if an otolith context cue (head orientation) can be used to switch between two different states of a reflex that is itself mediated by the otoliths (the LVOR).

Previous Studies

Before beginning the studies described here, using head position and LVOR gain, we studied vertical eye position as a context for LVOR phase adaptation. We performed a series of adaptation experiments to induce a phase lead in the LVOR when looking up, and a phase lag when looking down (or vice versa), in 4 subjects. A computer-generated visual display presented an image of a moving wall at a virtual distance of 1 m, with markers to set vertical eye position at 20 deg up and 20 deg. Sled motion was at 0.5 Hz, 0.3 g peak acceleration. During the first 3 min of adaptation, the visual-vestibular mismatch called for LVOR phase lag of 45 deg, while the subject looked 20 deg up. During the next 3 min, a phase lead of 45 deg was asked for, while the subject looked down 20 deg. This was repeated for 30 min (5 intervals up, 5 down). Adaptation time was limited to 30 min due to imminent overheating of the sled motors. Before and after adaptation, the LVOR was measured in the dark with the eyes up and down 20 deg, and centered. The subject was told to track the remembered location of a target at each vertical position.

Although not as robust as conventional LVOR gain or phase adaptation, there was after the adaptation session a tendency for phase changes to be associated with vertical eye position. These changes were small and dissipated quickly, but provided evidence that vertical eye position could be used as a context cue for adapted LVOR responses. Two subjects showed significant context-specific adaptive changes in phase. Another gave all positive (lead) phase changes, whether looking up or down; this subject differed from the others, who have natural phase leads and reliable smooth tracking in the dark. The final subject produced mixed results. These results were promising but severely limited due to the short adaptation duration (30 minutes) available on our sled at that time.

In a separate study we found that the gain and the phase of the LVOR can be adaptively modified after 20 minutes of ×1 stimulation at 0.5 Hz. The gain changes are not simply due to instantaneous changes in vergence (the gain would be expected to increase for near viewing), the gain changes were seen with step as well as sine stimulation, and catch-up saccades made up a significant component of the adaptive gain response but not the adaptive phase response.

In addition, we finalized the development of an algorithm for the analysis of the LVOR smooth tracking component, with saccades removed. This algorithm was used in the study just described, and has been described in another manuscript which has been submitted for publication.

Equipment

Progress on this subproject was significant in two areas, engineering and science. In the engineering area, we completed long-awaited modifications to our human linear acceleration sled.

These modifications involved extensive reworking of the air conditioning system in the sled room, to blow cold air over the sled motors continuously (heating of the sled motors had previously limited adaptation durations to 30 minutes or less). We also installed a set of springs to make the moving cart system resonant at 0.7 Hz. The electronic controller/computer system alone was not capable of driving the sled with reliable oscillations at this frequency (the controller update rate was too low). We are now able to run adaptation experiments for well over an hour (engineering tests have been run for over 3 hours), at a frequency of 0.7 Hz, where the translational LVOR is more reflexive and less dependent on smooth pursuit.

Procedures

Subjects were translated from side to side while seated upright on a linear sled. The sine-wave stimulus had a frequency of 0.7 Hz and a peak acceleration of 0.3 g (± 0.15 m displacement). The head was restrained during translations with a biteboard and pads pressed against the sides of the head. During sled motion, a visual display (poster board, 1.2 m horizontal by 0.9 m vertical) was presented, at a distance of 0.74 m from the subject's eyes. The display could be made to move either in phase with the sled (requiring no eye movement to compensate for body motion: an LVOR gain of 0), or it could be made to move out of phase with the sled (requiring twice the normal amount of eye movement to compensate for body motion: an LVOR gain of ×2). Eye movements were recorded with scleral search coils (in each eye) in a magnetic field, at a sample rate of 500 Hz. The coils were inserted for testing before and after adaptation, and removed for all but the last 5-7 minutes of adaptation.

Three experiments were performed, using head position as a context cue. In the first experiment, head roll was used as the context: with the head rolled 45 deg to the right shoulder a gain increase was called for (×2 stimulus), and with the head rolled 45 deg to the left shoulder a gain decrease was called for (×0 stimulus). In the second experiment, head pitch was the context: with the head pitched down 26 deg a gain increase was asked for, and with the head pitched up 26 deg a decrease was called for. The final experiment again used head roll as a context cue, but with 26 deg of roll, for more direct comparison with the results of the pitch experiment. Each experiment was performed with a one-hour adaptation period, alternating between the two gain conditions (and head positions) every 5 minutes.

A control experiment was performed in which we looked for gain increase adaptation only, with a one-hour adaptation period.

For all experiments, fast phases were removed from the data and gains and phases relative to sled motion were computed.

Results

Using head roll as the context cue (left side of Fig. 2.1), we found a substantial decrease in LVOR gain in each of three subjects with the head rolled to the left, as called for (ranging from 31 to 53%). We found varying, and on the average much smaller, amounts of gain increase with the head rolled to the right (from 4 to 36%), which was also called for by the adaptation procedure. Thus the gain had become context-specific. These results depended to some extent on the amount of head roll used as the context cue, with 45 deg roll more effective than 26 deg roll (although this analysis is in progress).

A control experiment was also run, in which the only adaptation imposed was ×0 with the head upright, while the gain was measured with the head rolled and upright; not surprisingly there was transfer of the gain-decrease adaptation to the roll condition.

Head pitch as a context cue did not work nearly as well, with one subject even showing adaptive changes in the opposite direction to those desired. (We should point out that this result appears to be counter to one we previously reported, in a NSBRI progress report. That report was

based on preliminary data and the current one was done more carefully and on more subjects; nevertheless the discrepancy is being investigated, and may be due to differences in the removal of fast phases from the compensatory slow phase responses.)

A control experiment was also run, in which the only adaptation imposed was ×2 with the head upright. Surprisingly, this appeared to produce no gain change or even a decrease in gain. Adaptation to decrease the gain of the LVOR is more successful than adaptation to increase the gain. This helps to explain the asymmetry in gain adaptation in our context-specific adaptation experiments.

Our results show that the use of gravity as a context cue for otolith-mediated responses is indeed possible. Head pitch is less effective than head roll, for LVOR stimulation along the inter-aural axis – the same axis stimulated by roll head tilt. This suggests that to be effective a context cue must be closely associated with the response being adapted.

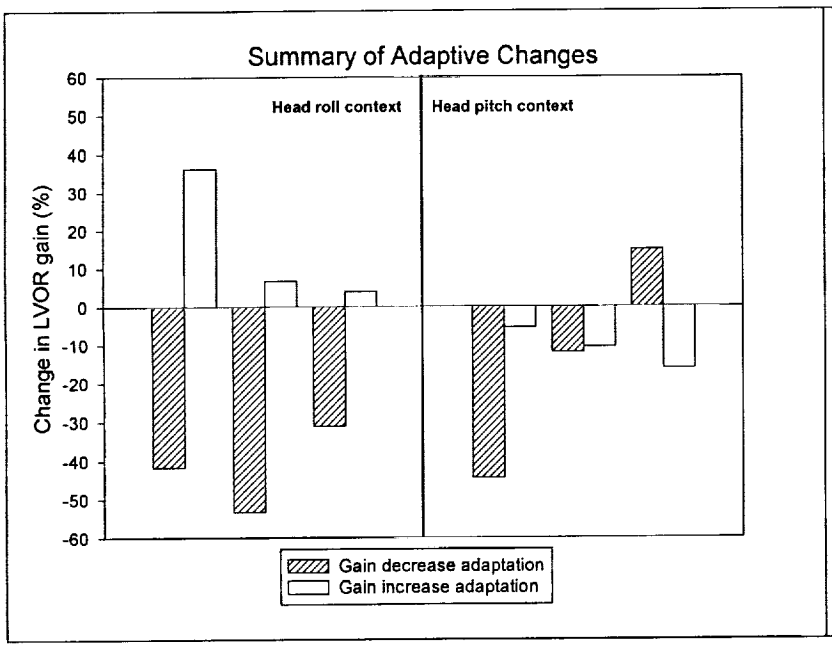


Figure 2.1. Summary of context-specific LVOR adaptation experiments. Each pair of bars represents a single experimental session, in which LVOR gain was adapted upward in one context and downward in another context, with the contexts alternating over the course of the session. To the far left are sessions using head roll (45 deg) as a context cue; to the right, head pitch (26 deg) was used as a context.

Aim 3: Context-specific Adaptation of Oculomotor Responses to Centrifugation

PI: Young. Assisted by H Hecht, C Cheung, K Sienko, L Lyne, J Kavelaars, A Natapoff.

Introduction

Traditional countermeasures against the adverse effects of prolonged weightlessness, such as exercise, resistive garments and lower-body negative pressure, appear to be insufficient in practice and are often disregarded by astronauts. Artificial gravity represents a potential countermeasure that is unique. Rather than alleviating the symptoms, it attempts to remove their cause. Although long a favorite topic of scientists and science fiction authors, it is only now receiving serious attention for space flight experiments and validation (Young 1999). Recent task groups and countermeasure workshops conducted by NASA and the National Space Biomedical Research Institute (Paloski & Young 1999) have refocused attention on the potential use of artificial gravity (AG) as an in-flight countermeasure. It could be effective against bone and muscle loss, cardiovascular deconditioning, and neurovestibular disturbances. Unfortunately,

space limitations within existing and planned vehicles demand that any AG centrifuge device tested in the foreseeable future be of limited radius (on the order of 1-3 meters). Centripetal accelerations equal to or exceeding 1 g will therefore require relatively high angular velocities (on the order of 30 rpm). Consequently, out-of-plane head movements on the centrifuge will create new, unexpected semicircular canal inputs producing illusory sensations, inappropriate non-compensatory vestibulo-ocular reflexes, and motion sickness. Thus, practical use of an intermittent short-radius centrifuge for in-flight gravity treatment requires that crewmembers be capable of rapidly adapting to the unexpected canal inputs with minimal side-effects or after-effects. Furthermore, it will be essential to retain the astronauts' adaptation to the 0 g state in order to avoid "Space Adaptation Syndrome" each time they make a transition from the centrifuge to weightlessness. Our recent findings obtained with the MIT short-radius centrifuge (SRC) encourage the use of an SRC as a viable countermeasure.

After rotation for more than about 30 seconds the cupulae of the canals return to the neutral position and the sensation of rotation disappears. Out-of-plane head movements executed while spinning at a constant angular velocity, move the semicircular canals into and out of the plane of rotation. The endolymph in the canals deflect the cupulae, sending signals to the brain which no longer correspond to the expected head velocity signal produced during head movements in a non-rotating environment (Guedry 1974, Young 1983). The discrepancy produces illusory body tilt and causes motion sickness. It also triggers an inappropriate angular vestibulo-ocular reflex (AVOR) about an axis perpendicular to both the axis of head movement and the AG spin axis.

Consider a subject lying supine on a radially aligned bed rotating about a vertical axis. During a yaw head turn in a non-rotating environment, the vestibular system receives a pure yaw input. However, that same head turn conducted within a clockwise rotating environment (angular velocity vector of the centrifuge perpendicular to the head yaw axis) produces additional canal stimulation in the pitch and roll planes. These signals produce an inappropriate non-compensatory eye response in the vertical direction with respect to the yaw head movement. At slower rotation rates, up to 6-10 rpm, subjects adapt to these side effects over several days of activity (Guedry 1965). However, the higher rotation rate required for short-radius countermeasure purposes produces much more disturbing side effects.

We summarize here several studies performed on the MIT short-radius centrifuge (SRC), which are written up in detail in two attached manuscripts (Appendix B: Young et al. 2000, Hecht et al. 2000). The main study investigated adaptation to repeated head movements while rotating. We examined the vestibulo-ocular reflex (VOR), illusory tilt, heart rate and motion sickness (adaptation study). The second study focused on assessment of illusory tilt, motion sickness and heart rate of 20 subjects while they made out-of-plane head movements (assessment study).

Main adaptation study: Evidence for context-specific adaptation

We have demonstrated that context-specific adaptation can be achieved for head movements in a Coriolis environment. Eight subjects participated in an experiment that included three adaptation sessions. During each session, subjects were placed supine on the MIT rotating bed (SRC), head on the center of rotation and feet outboard. The bed rotated at 23 rpm in a clockwise direction, producing 1 g at the feet of a 168 cm tall person. Vertical and horizontal eye movements as well as illusory body tilt were induced by yaw head movements made in the dark. Then, during a 10 min adaptation period in the light, with the visual surround fixed to the rotating bed, subjects made as many yaw head movements as possible. Yaw head movements were always from right-ear-down (RED) to nose up (NU) and back. Each 90° head movement had to be executed within 1 sec. It was assumed that because the head movements were being

made in the presence of a lighted background, the oculomotor system would become "re-educated" to the novel vestibular condition. Finally, another set of measurements was taken while rotating in the dark. This procedure was repeated the following day and a week later.

These head movements produce cross-coupled angular accelerations and, more importantly, change the position of the semicircular canals with respect to the plane of rotation, which causes unexpected acceleration/deceleration of the endolymph in the canals. The canal signal caused by a head movement no longer corresponds to the expected signal that is produced in a non-rotating environment. The discrepancy provokes motion sickness and the sensation of illusory body tilt. It also triggers a VOR in a direction perpendicular to the plane of the head turn (in our case a vertical nystagmus).

For instance, upon a counterclockwise yaw head movement from RED to NU, the subject should feel a whole body pitch forward and a clockwise rotation. The pitch forward is induced by relative motion of the endolymph in the canal that is taken out of the plane of the centrifuge's rotation. (Both the anterior and posterior canals contribute to the sensation.) During sustained constant rotation the cupulae had returned to the neutral position, indicating a cessation of rotation. Immediately after a head turn the cupulae continue to be stimulated, but the head is now in a new (NU) position. The inappropriate canal signal (forward pitch) is perceived and also "compensated" by vertical eye movements (inappropriate nystagmus). The hypothetical roll canal (posterior and anterior components), which had been outside the plane of rotation, is now in that plane and its endolymph responds to the angular acceleration of the bed's rotation. This results in a perceived rotation in the same direction as the bed (clockwise roll). The third (lateral) semicircular canal remains perpendicular to the axis of rotation and does not signal anything other than the actual yaw head movement.

We have measured the strength of the inappropriate vertical nystagmus, motion sickness, and the amount of illusory tilt. Not only did all three measures decrease from the beginning to the end of the adaptation period in the light, they continued to do so on the second day. Motion sickness was assessed using an on-line rating scale from 0 (fine) to 20 (about to vomit) and by means of a Pensacola assessment at the end of the experiment. Both measures showed clear adaptation. Figure 3.1 shows significant (N=8) adaptation of the inappropriate nystagmus over 3 days of practice, as measured by the averaged slow-phase velocity component (SPV) of vertical eye movements. Figure 3.2 depicts the raw SPVs for a typical subject.

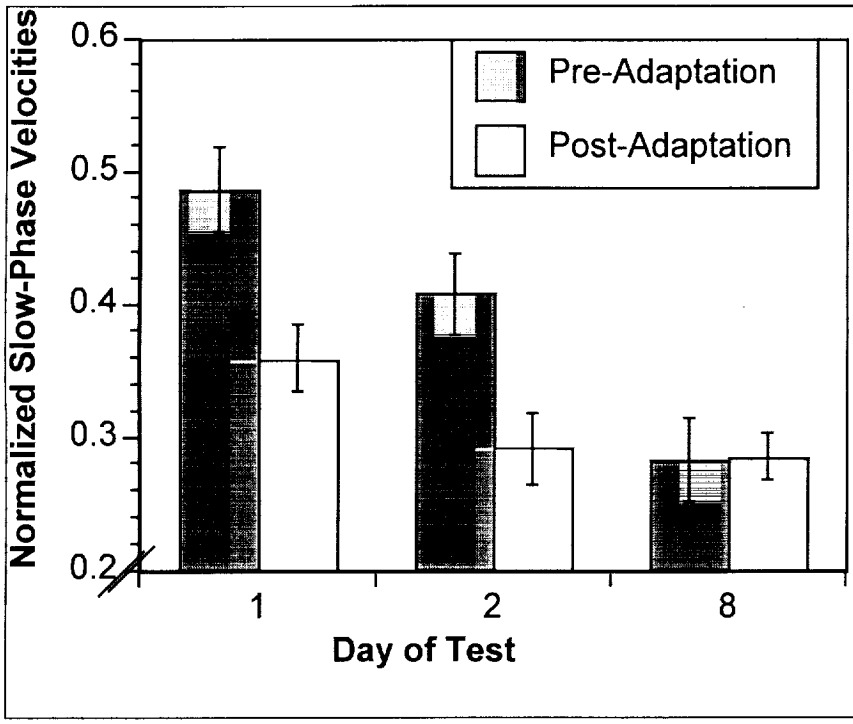


Figure 3.1. Adaptation study: Normalized slow-phase eye velocities measured in the dark before and after the 10-min adaptation period in the light. The normalization accounted for variations in head movement velocity. The values reflect averages of the second and third set of head movements to the right. The very first head movements show very little adaptation, suggesting a feed-forward context cue for adaptation. Error bars indicate standard errors of the mean.

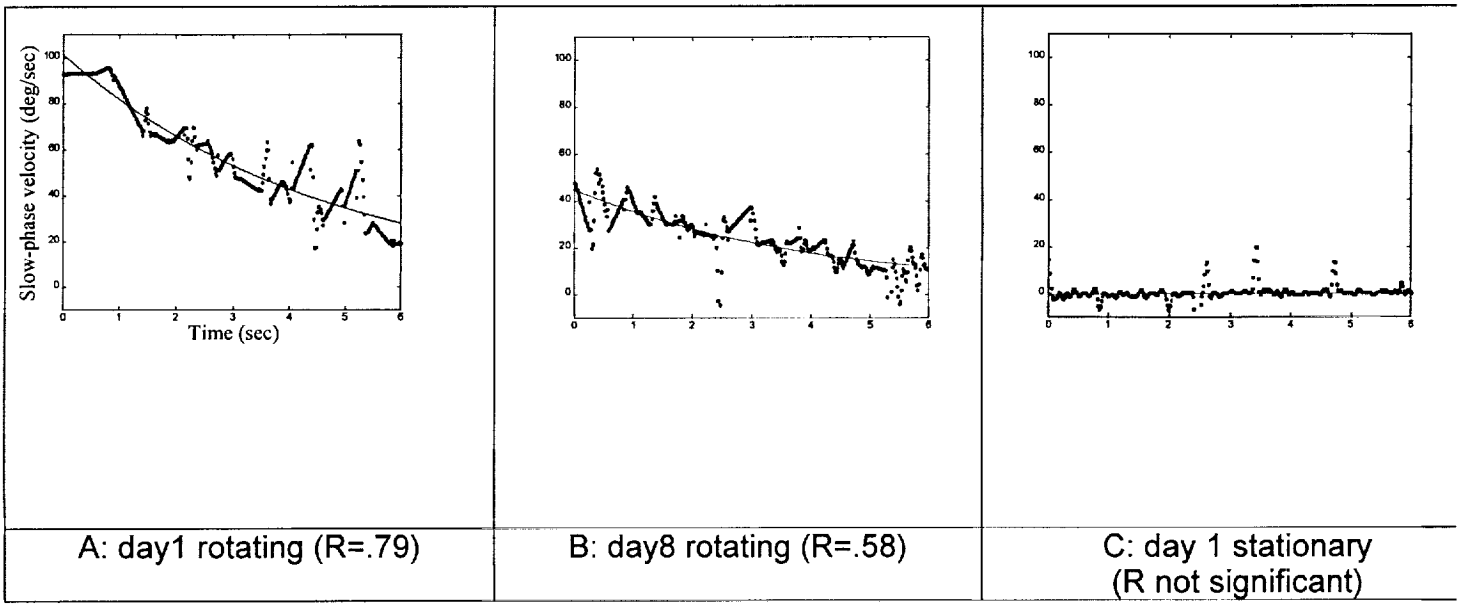


Figure 3.2. Adaptation study: Raw slow-phase velocity profiles, in deg/sec for one subject (S6) for head turns from RED to NU (second repetition). Panel A: before adaptation while rotating. Panel B: after adaptation while rotating. Panel C: Before adaptation while making a head turn on a stationary bed. It is very important to note that the adaptation revealed in the session-to-session reduction of pre-adaptation nystagmus is context specific, because no oscillopsia or nystagmus were present between sessions outside the SRC.

The results also indicate that adaptation is not easy to achieve and that subjects continue to adapt after two sessions. Most, but not all, of the adaptation was retained after 7 days. This adaptation was context-specific since no measurable effects were observed after subjects left the centrifuge. Eye movements and perceived body orientations were normal upon head movements in the stationary environment, as seen in Figure 3.2 (Panel C). However, at least a part of the adaptation consisted in a generalized suppression of the VOR, as was determined by a decrease of the cumulative slow phase eye movements during acceleration of the centrifuge at the beginning of the experiment and its deceleration at the end. Those accelerations were never experienced during the light adaptation period.

Assessment study: Subjective responses to a cross-coupled vestibular stimulation in different planes

First, the existing MIT short-radius centrifuge had to be modified and instrumented for the purposes of our study. A Watson 3-axis sensor system was selected to measure head movements because of its light weight, low power, high sampling rate, and low cost. To record eye movements, an ISCAN miniature eye imaging system was acquired. The ISCAN system was selected because it was light weight, comfortable for the subject to wear, did not restrict head movements, rivaled coil resolution, and had low drift characteristics. Also, because of its infrared imaging system, it is possible to record eye movements in complete darkness, which is required for all control measurements.

We tested 20 observers, who were asked to execute a variety of yaw and pitch head turns while supine on the rotating centrifuge. All observers reported head-contingent sensations of body tilt although their bodies remained supine. Most of them did experience the predicted pitch and roll during yaw turns and yaw and roll sensations during pitching head movements. However, a small but significant percentage of observers reported tilt in the predicted plane but in the opposite direction. The inappropriate nystagmus measured in Experiment 1, however, was always in the plane and direction predicted by the semicircular canal model. Figure 3.3 shows the duration of illusory tilt sensation, which we measured to assess subjective strength of the aversive illusion. Head turns from ear down to nose up generally produced longer lasting pitch illusions than in the opposite direction. Mostly, the subjective sensations conform to a model based on semi-circular canal responses to angular acceleration. However, some surprising deviations were found for head movements in the pitch plane. Also, large inter-individual differences in direction, magnitude, and quality of the illusory body tilt were observed. The results have implications for subject screening and prediction of subjective tolerance for centrifugation.

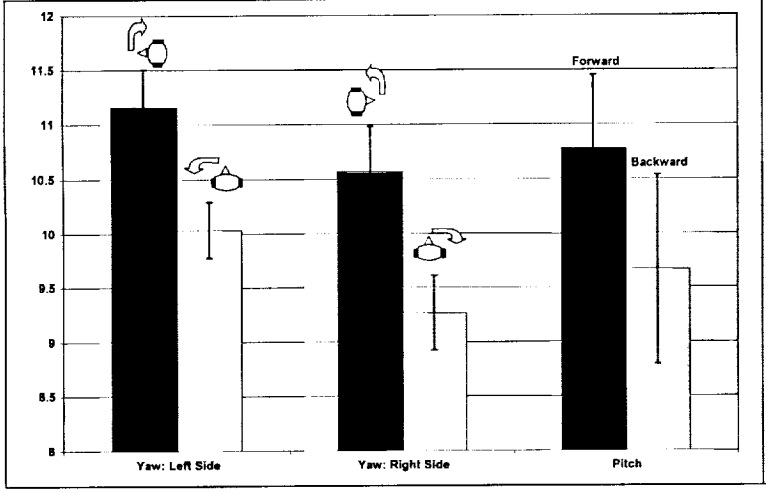


Figure 3.3. Assessment study: Duration of illusory tilt sensation following initiation of head turn (in seconds)

Qualitative assessment of illusory tilt:

Two measures were computed for subjective tilt, illusory motion (magnitude and direction) and its duration. Although all 20 subjects made three sets of yaw head movements to each side, only 15 reported motion sickness scores below the criterion (score < 3) that allowed them to perform additional pitch head movements. The data consisted of audio tape and written recordings of verbal reports while subjects were on the centrifuge. Ambiguous reports were followed-up after the rotation period by asking the subject to demonstrate perceived motion using a puppet.

For yaw head movements, the majority of subjects experienced the predicted pitch and roll directions. However, a few subjects perceived illusory motion in the predicted plane but in the opposite direction. This was the case in 13% of all yaw head-turns. This direction inversion was inconsistent insofar as it occurred mostly for illusory pitch. Also, those subjects who reported inverted directions did not do so consistently.

In the case of pitch head movements, 40% of the time, the 15 subjects reported an additional body tilt in the direction of the head movement. This is inconsistent with the canal model. Subjects clearly reported a strong full body pitching sensation, which could not have been confused with the comparatively small actual pitch head movement. Tables 3.1a and 3.1b summarize the illusory tilt sensations for yaw and pitch head movements respectively and compare them to the predictions of the semicircular canal model.

Subjective Motion	**Predicted Direction**	**Opposite Direction**	**Corresponding To Actual Turn**	**None**
Pitch	76 %	13%	-	11%
Roll	60%	13%	-	27%
Yaw	89%*	-	11%	89%*

Table 3.1a. Yaw head movements: Percentages of reported illusory body motion while rotating in a supine position. *Note that illusory pitch and roll body motions are predicted while yaw is not.

Subjective Motion	**Predicted Direction**	**Opposite Direction**	**Corresponding To Actual Turn**	**None**
Pitch	60%*	0%	40%	60%*
Roll	53%	0%	-	47%
Yaw	43%	7%	-	50%

Table 3.1b. Pitch head movements: Percent of illusory motions reported while rotating in a supine position. *Note that illusory pitch body motion is not predicted.

In summary, the above studies have demonstrated that context-specific adaptation of eye movements and experienced body-tilt do in fact occur. They also underline the importance of understanding the mechanism of adaptation and its degree of specificity.

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Aims 4 & 5: Properties and Context-specificity of Vestibulocollic Reflex

PI: Goldberg. Assisted by WH Paloski, S Lutes, S Wood.

Introduction

Sensors located in the vestibule of the inner ear signal motion of the head and its orientation relative to gravity. Changes in gravito-inertial forces (gif) during space travel alter these vestibular signals and impair balance, vision and spatial orientation. This study of the head-neck system follows our overall strategy for determining human capacity for adaptation to altered gif and vestibular inputs. Its first aim is to quantify head oscillations induced by body motion in three dimensions and determine effects of vestibular inputs on these oscillations. The second aim is to test the capacity of the system for context-specific adaptation to altered gif environments. The ultimate goal is the development of countermeasures to changes in gif so that optimal performance of the system can be maintained during space travel and planetary exploration.

Head motion has been implicated in the genesis of space motion sickness. Since the head serves as the platform for visual and vestibular receptors involved in perception of motion and spatial orientation, excessive head oscillations induced by body motion can cause reduced visual acuity, disorientation, and poor balance. The head-neck system must be capable of limiting oscillations to prevent such undesirable effects of motion. This requirement is especially important for vision in unusual gif environments, where excessive oscillations may exacerbate the problems created by an inadequate vestibulo-ocular reflex (VOR). Neck muscles tend to act on the head in the manner of springs and shock absorbers of a car. However, these passive mechanical properties of the neck cannot prevent oscillations of the head because of its relatively

large inertia. We hypothesize that active contractions of neck muscles in response to dynamic vestibular inputs provide the needed additional damping. This mechanism and brain pathways by which vestibular inputs act on neck muscles is referred to as the Vestibulo-Collic Reflex (VCR).

Vestibular inputs to the VCR from the semicircular canals signal head rotation in space, and those from the otoliths signal head translation in space, as well as its orientation with respect to gravity. Head rotation in space is the sum of trunk rotation in space and head rotation relative to the trunk (neck rotation). VCR induced by stimulation of semicircular canals during trunk rotation tends to rotate the neck in the direction opposite of the trunk rotation. Thus the canal-induced VCR acts to reduce head rotation in space and decrease its input. This action of the VCR can be represented by negative feedback in a "closed-loop" model.

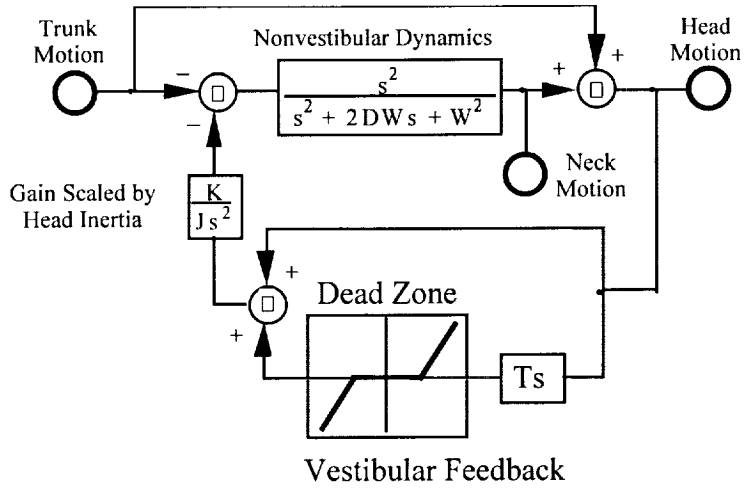


Fig 4.1. A model of head/neck dynamics in the yaw axis.

Figure 4.1 illustrates a model of the head-neck system based on these considerations and data we obtained previously. The model was devised to account for head rotation responses induced by trunk rotations about a vertical (yaw) axis passing through the neck. The block with the second-order transfer function labeled "Nonvestibular Dynamics" represents the input-output properties of the head/neck mechanics (plant) alone. The neck rotation output of the plant is converted to head rotation in space by the addition of trunk rotation via the feed-forward path. The VCR is represented by the vestibular feedback loop around the plant. The plant transfer function is characterized by the "open-loop" damping ratio (D) and natural frequency (W) parameters. Since D is on the order of 0.05, the plant is quite underdamped. Damping is increased by the rate feedback arm of the VCR, which consists of the differentiator (s), lead time constant (T), and threshold nonlinearity (Dead Zone). This arm represents a phase-advanced signal presumably originating in high-threshold, irregular canal afferents. Because of the threshold, the effective damping factor depends on input amplitude, in accordance with actual data (for illustration, see http://www.bcm.tmc.edu/cfbd/NSBRI).

For amplitudes well above threshold, the VCR feedback arm can be approximated by a linear block (Ts+1). The model can be then represented by a simple transfer function with a second-order denominator and a first order numerator, with effective D and W dependent on VCR feedback parameters, T and K. A similar model can be derived for head rotations induced by trunk translations. In this case, the model would not have the feed-forward arm of Fig. 4.1, since

the trunk does not rotate. The resulting transfer function then would have both a second-order numerator and denominator.

As will be seen below, head rotation responses to body motion may be three-dimensional, even if the stimulus motion is confined to one axis. It is important to determine whether such responses can be characterized in terms of relatively simple models, similar to the one described above. If this is possible, we should be able to infer the role of vestibular feedback in adaptation from changes in input/output characteristics of the head-neck system.

Head-Neck system dynamics in three dimensions

Spatial and dynamic characteristics of head rotation responses to body motion have not previously been systematically analyzed in three dimensions. We have been testing these responses as a part of the first aim of this sub-project. A series of experiments has been carried out using the short-arm centrifuge (SAC) facility at the Johnson Space Center. The SAC can be configured to apply pure rotation or translation combined with rotation. Subjects were tested while seated upright, with the vertical rotation axis in each of three positions relative to the neck: (a) centered, (b) 0.8 m behind or (c) 0.8 m to one side. These configurations are illustrated in the bottom row of Figure 4.3. Motion stimuli followed sum-of-sines waveforms with components in 1-3 Hz and 4-10 Hz ranges, while subjects performed mental arithmetic in the dark. Responses to the 1-3 Hz range were used for analysis described below, because they had low noise and covered the critical dynamic range. Rotational velocity sensors on the head and chest were used to record head and trunk motion in 3-D. Figure 4.2 shows our coordinate system for the components of head rotation velocity. The same system was used for trunk motion.

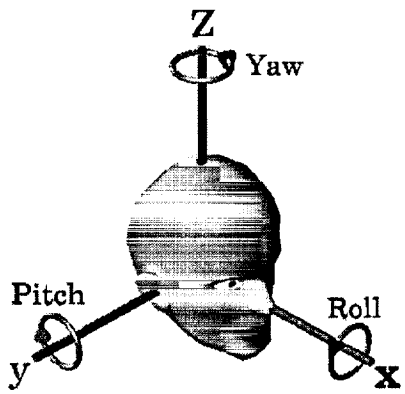


Figure 4.2. Coordinates of head rotations.

Figure 4.3 summarizes spatial components of the stimuli and responses in the three configurations. The bars represent means and standard errors of root-mean-square (RMS) rotation velocity in 17 subjects. Note that the trunk stimulus contains a large yaw component in each configuration. In the two eccentric configurations, this component is also a proxy for a large translational stimulus component. In addition to these intended, primary stimulus components, trunk rotations contain several smaller, unintended components. Some of these secondary components appear to be are related to the primary ones and are likely due to imperfect trunk restraint. Each of the three head rotation responses in the upper panels has 3-D components.

Additional experiments showed that some of the components are at least partially due to head-neck spatial properties rather than secondary stimulus components.

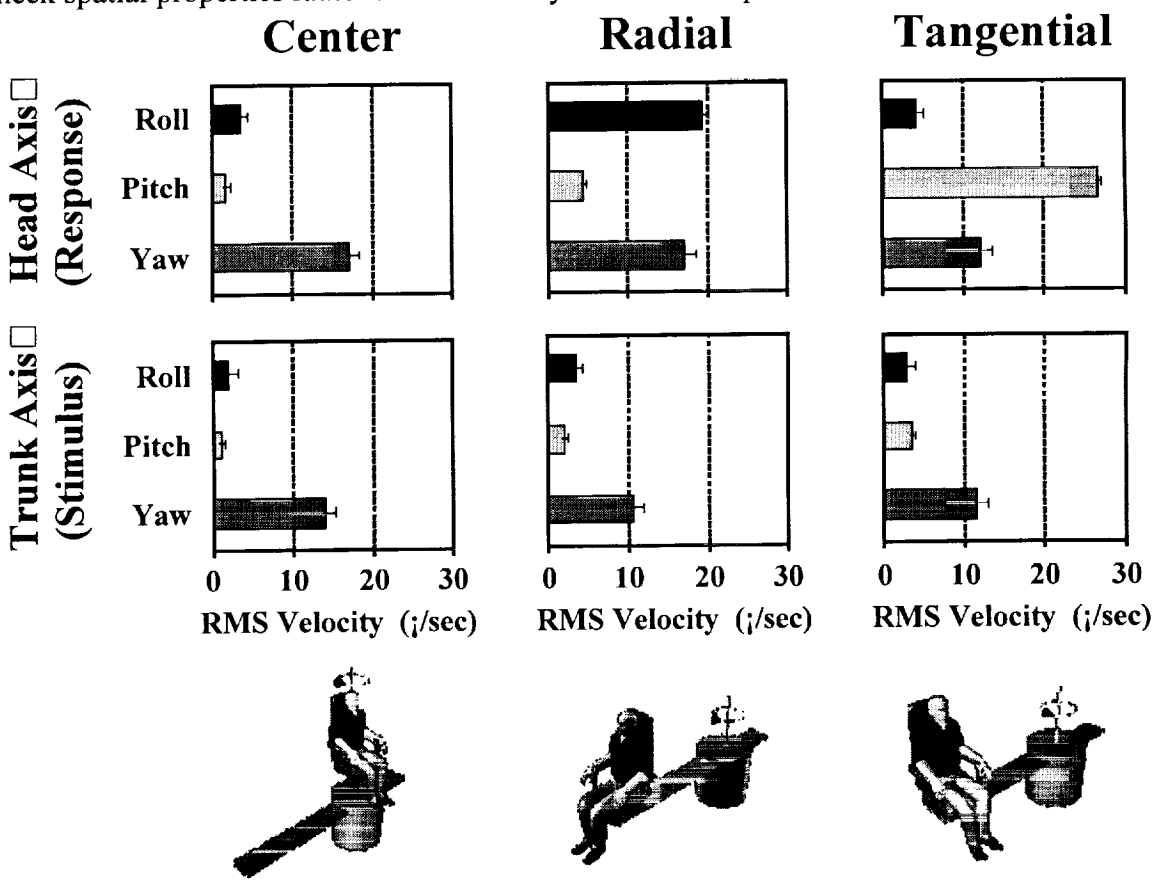


Fig 4.3. Amplitudes of stimulus and response components.

Can these seemingly complex responses be related to the stimuli in terms of simple models? We have been carrying out a systematic analysis of model complexity required to explain each response component (output) in terms of one or two stimulus components (inputs). The results are briefly summarized in Figure 4.4. For each configuration, the three table columns correspond to three components of the output. The first row lists one or two inputs that provide the best fit. The second row gives the corresponding order(s) of the transfer function numerator (the denominator is always of second order). The third row gives the percent of variance explained by the model. These results suggest that most of the 3-D response dynamics can be explained reasonably well by relatively simple models.

	Center			Radial			Tangential		
	Roll	Pitch	Yaw	Roll	Pitch	Yaw	Roll	Pitch	Yaw
Trunk Inputs	Roll, Yaw	Roll, Yaw	Yaw	Roll, Yaw	– –	Roll, Yaw	Roll, Yaw	Pitch, Yaw	Yaw
Input Orders	1, 2	1, 2	1	1, 2	– –	1, 2	1, 2	1, 2	1
Avg R^2 (N=17)	0.87	0.51	0.98	0.68	0.5 <	0.76	0.53	0.67	0.88



Figure 4.4. Summary of simplest second-order models that fit response components.

Adaptation of head-neck responses to increased moment of inertia

An artificial increase in head inertia causes an immediate increase in head oscillations induced by trunk motion. We tested the adaptive capacity of the head-neck system for reducing the amplitude of such oscillations. Head rotation responses were induced by trunk rotation about the yaw axis. Sum-of-sines rotations in the 0.55-4.15 Hz range were used for testing and adapting these responses in 14 subjects. Head moment of inertia in the yaw axis was increased from its baseline value, J_0 (head + lightweight helmet) to $5 \times J_0$. Adaptation was induced by rotating the subjects for 20 minutes at 24 deg/sec RMS, while they watched television. The run was then repeated with inertia reset to J_0, in order to induce re-adaptation back to baseline.

Subjects were tested in the dark before and after each adaptation run at the two inertia levels and two stimulus levels: "large and "small". The RMS amplitude of the larger stimulus was 24 deg/sec in five subjects and 12 deg/sec in the other nine (the results were similar between the groups). The amplitude of the smaller stimulus was 6 deg/sec in all subjects. Sensors on the trunk and helmet recorded yaw angular velocities. RMS head velocity served as the measure of response amplitude. This amplitude was expressed as the percentage by which it differed from its baseline value obtained with J_0 for each subject and test condition.

The four panels in Figure 4.5 summarize response amplitudes obtained under the four test conditions before and after adaptation to $5 \times J_0$ inertia. The symbols and vertical bars represent means and SDs (N=14) of the amplitudes relative to baselines. Panel A shows that before adaptation, the response amplitude at the $5 \times J_0$ inertia level averaged 5.7% above baseline. Adaptation decreased this amplitude by an average 14.5% and thus brought it below baseline. Panel B shows that at the small stimulus amplitude, the reduction was smaller than in A and brought the RMS amplitude only halfway toward baseline. The adaptation was thus dependent on stimulus amplitude, suggesting that it involved the VCR. The even smaller reductions seen in Panels C and D indicate that the adaptation was somewhat specific to the $5 \times J_0$ inertia level.

Tests after re-adaptation to J_0 showed that responses obtained with the original inertia J_0 were brought closer to baseline. However, the responses obtained with $5 \times J_0$ were not affected, indicating the persistence of the original adaptation to $5 \times J_0$.

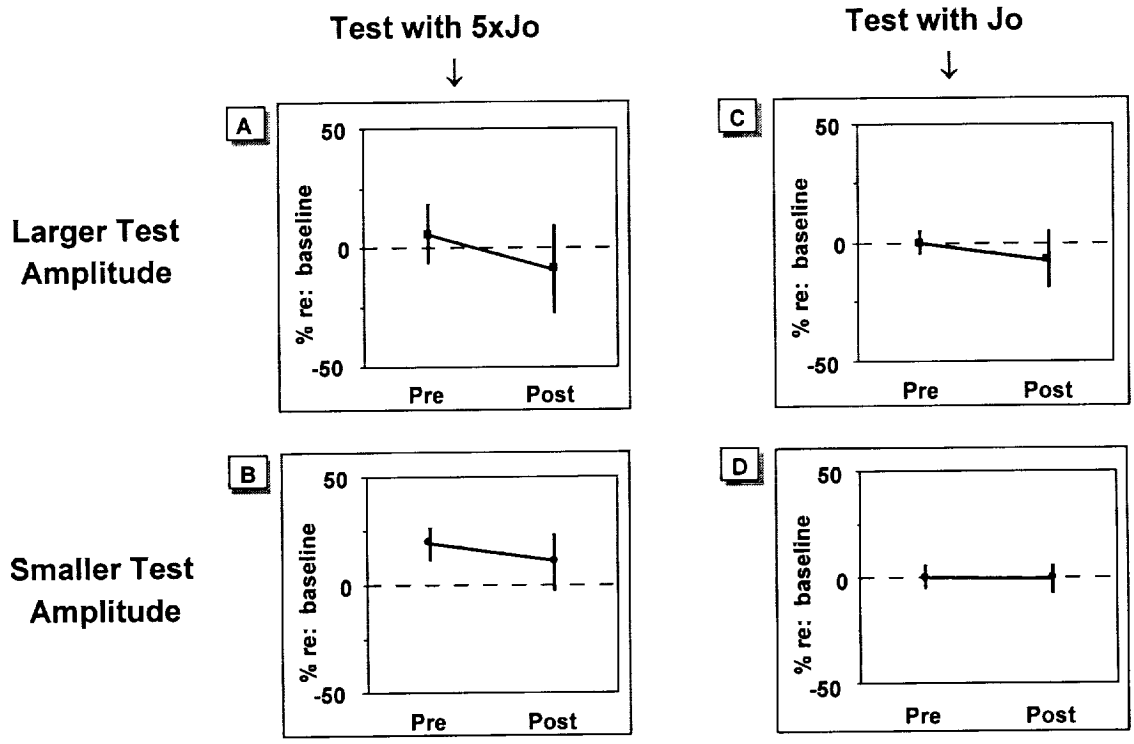


Figure 4.5. Effects of adaptation to 5 x J_0 on response amplitudes.

Fig. 4.6 illustrates changes in oscillation amplitude of one subject tested as part of a pilot study. On the left, the initial increase in amplitude caused by the 5× inertia is the distance between the 100% (baseline) and 127% points at time 0 min. The amplitude decreased toward the baseline during the 30-min adaptation run. Follow up testing, illustrated on the right of Fig. 4.5, indicates that the subject remained adapted to 5× head inertia even 35 days later. The results also suggest that the head-neck system is capable of dual adaptation and tends to maintain roughly the same level of oscillations in both 5× and normal inertia contexts.

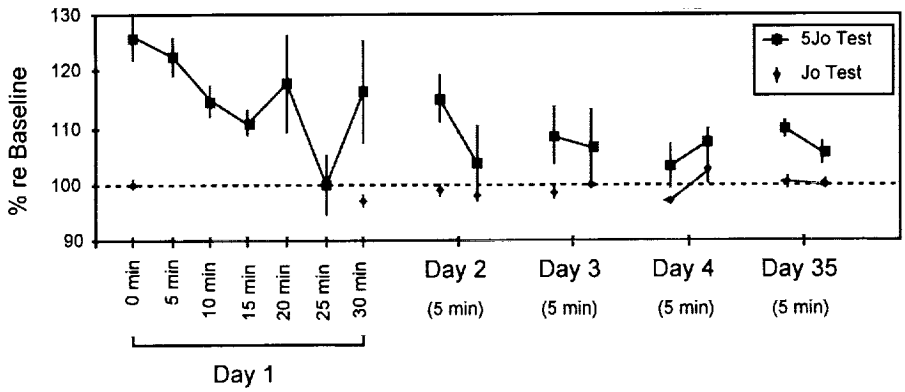


Figure 4.6. Acquisition, retention, and specificity of adaptation to a 5× head inertia increase. Large symbols, lines and SD bars indicate changes in head oscillation amplitude during initial 30-min adaptation on Day 1 and repeated 5-min adaptations on Days 2-35. The head inertia was 5× normal (5Jo) for these measurements and adaptation. All symbols represent RMS head velocity expressed as % of baseline. The baseline measurement was obtained with normal inertia (Jo), just before adaptation, indicated by the small symbol at 0 min, 100%. The same small symbols also indicate the relative constancy of oscillation amplitude tested with Jo at the other times. Head oscillations were induced by sum-of sines (0.55-4.15 Hz) trunk rotations in horizontal plane. The subject began complaining of pressure from the tightly-fitting helmet 20 minutes after start of the initial 30-min adaptation.

Our results show that the head-neck system can adaptively reduce oscillations in an altered inertial environment. The adaptation appears to be specific to inertial context and persists after the subjects are re-adapted back to normal inertia. Thus, after the "dual" adaptation, the system responds appropriately to each inertial load and keeps head oscillations at the same level.

Aims 6, 7, 8: Cerebellar Contribution to Context-specific Adaptation in Monkeys

A. PI: Minor. Assisted by R Clendaniel.

Introduction

A set of experiments in squirrel monkeys was carried out to examine the translational LVOR and its adaptation, and to more fully elucidate the characteristics of the angular VOR and its adaptation. Experiments to date have investigated adaptation of the gain of the translational LVOR and looked for transfer of adaptation to the oculomotor response to tilt, and have studied the basic properties of the AVOR, in particular linear and nonlinear neural pathways and their behavior under adaptation.

Equipment and Procedures

A rotation-translation-tilt apparatus is used for the testing of squirrel monkeys. This apparatus has three independently servo-controlled axes of motion. The base axis is a direct drive rotation motor with a torque of 300 ft.-lb. Attached to this base axis is a belt-driven linear sled with an excursion of 1 m. The third axis controls tilt about a plane centered through the animal's head. Rotational stimuli at sinusoidal frequencies of up to 15 Hz and peak velocities of 100 deg/s at 4 Hz and 25 deg/s at 15 Hz can be delivered with the animal positioned on the center of the axis of rotation. Rapid changes in head velocity with accelerations of up to 3000 deg/s^2 can also be given in this position. These peak performance characteristics diminish when the device holding the animal and associated field coil apparatus is positioned eccentrically, but the system can still deliver rotations of up to 10 Hz at a peak velocity of 40 deg/s for sinusoids and steps of acceleration up to 1500 deg/s^2 when this device is positioned 50 cm eccentric to the axis of

rotation. These characteristics are ideal for generating stimuli that involve combinations of angular and linear acceleration.

The linear sled can produce sinusoidal translations at frequencies of up to 4 Hz and peak accelerations of 0.5 g. Steps of acceleration of up to 0.8 g can also be delivered. The tilt axis can rapidly position the animal into a 180 deg head orientation with either ear down with respect to gravity. It can also be used to oscillate the animal in pitch or roll at frequencies of up to 2 Hz at 20 deg/s. Three-dimensional eye movements are recorded with a search coil system that has three orthogonal magnetic fields. Horizontal, vertical, and torsional eye movements are transduced unambiguously with a position resolution of 0.02 deg.

Major portions of this apparatus were funded by NSBRI, and significant effort was expended in their installation during the term of grant support.

Results

We have begun a study of the adaptive capabilities of the linear vestibulo-ocular reflex (LVOR) in order to 1) see if the LVOR gain can be adapted, and 2) determine if there is transfer between the LVOR and the ocular tilt reaction (OTR). We induce adaptation by using magnifying goggles (2.2× lenses) and minimizing goggles (0.45× lenses). In these initial experiments we adapted the monkey with either a 1 Hz stimulus (peak acceleration 686.92 cm/s^2) or a 0.2 Hz stimulus (peak acceleration 98.03 cm/s^2) for 2 hours.

Sensitivity of the LVOR (tested in the dark) was compared at a number of frequencies before and after adaptation. Following adaptation with the 2.2× lenses at 1 Hz we observed an increase in the peak slow component eye velocity response of the LVOR when tested at 1 Hz (pre-adaptation: 39.96°/s, post-adaptation: 45.54°/s; $p<0.05$). Likewise, after adaptation with the 0.45× lenses we saw a decrease in the slow component eye velocity response of the LVOR when tested at 1 Hz (post-adaptation: 27.82°/s, $p<0.01$). When we tested the monkey at 0.5 Hz, we observed a decrease in response after adaptation with both the magnifying and minimizing goggles. When we adapted the monkey using a 0.2 Hz stimulus, we observed decreased responses of the LVOR at all testing frequencies (1, 0.5, and 0.2 Hz), regardless of the adaptation condition (magnifying or minimizing lenses). Following adaptation of the LVOR, there was no significant change in the torsional eye movements to head tilt, suggesting that the responses to head tilt and head translation are not tightly coupled.

Adaptation of the angular vestibulo-ocular reflex (AVOR) gain to both magnifying (2.2×) and minimizing (0.45×) lenses was studied using sinusoidal rotations and impulse testing in 6 squirrel monkeys. During the impulse testing, the gain of the AVOR was measured during both the acceleration (up to 3000°/s^2) and constant velocity (up to 150°/s) portions of the stimulus. Following magnification of the AVOR, the gain during the acceleration portion (G_A) was essentially identical to the magnification power of the lenses. The gain during the constant velocity portion (G_V) was greater than normal, but less than the magnification power of the lenses. Following adaptation with the minimizing lenses, both G_A and G_V were reduced symmetrically (Fig. 6.1). The post-adaptation AVOR gain during sinusoidal rotations was similar to G_V. Prior work in the lab has described a non-linear and linear component to the AVOR. Following adaptation with the magnifying lenses, the AVOR can be modeled as a selective gain enhancement of the non-linear component as compared to the linear component. Following adaptation with the minimizing lenses, the AVOR can be modeled as a decrease in the gain of the linear component and a "shutting off" of the non-linear component.

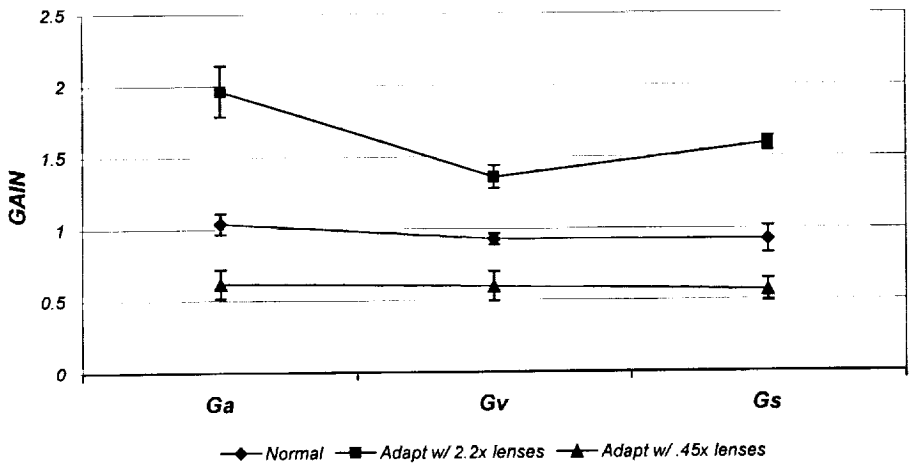


Figure 6.1. Gains of the AVOR pre-adaptation, post-adaptation with 2.2x magnifying lenses, and post-adaptation with 0.45x minimizing lenses. Ga is the gain during the acceleration component of a velocity step stimulus, Gv is the gain during the constant velocity component of the velocity step stimulus, and Gs is the gain during 0.5 Hz, 60°/s peak velocity sinusoidal stimulus.

B. PI: Zee. Assisted by M Walker, M Takagi.

The vestibulocerebellum is important for adaptive control of the AVOR, but little or nothing is known of its role in adaptive control of otolith-ocular responses. A set of experiments in rhesus monkeys is designed to determine the role of the vestibulocerebellum in the LVOR (the oculomotor response to translation), and in context-specific adaptation. The specific experiments described here are also designed to test the contribution of smooth pursuit eye movements to the generation of the translational LVOR.

Introduction

The main objective of this component of our research project is to use the rhesus monkey model, and human responses, to understand better the adaptive control of otolith-mediated vestibulo-ocular reflexes, and the consequent implications for human performance in altered gravitational environments. In many ways, the rhesus monkey is ideal for studying adaptive control, because of the flexibility that the experimenter has in designing both the type of learning paradigm and the protocols for its investigation (e.g., number, duration, and pattern of training sessions). The vestibulocerebellar-lesioned monkey is also a critical part of the focus here, both to better understand relationships among the various types of linear vestibulo-ocular reflexes (low- and high-frequency translation and tilt-induced counter-roll) and also because of the important finding that human astronauts subject to long-term microgravity show "cerebellar" ocular motor abnormalities, after extended flight.

The vestibulocerebellum may be particularly important for understanding the translational VOR because of its critical role in generating pursuit eye movements. Cerebellar lesions of the floccular/parafloccular region lead to severe impairment of smooth pursuit. Phylogenetically, smooth pursuit and the LVOR both become especially important with the evolution of a fovea, binocular, frontal-eyed vision, and the ability to point both eyes simultaneously at a specific location in three-dimensional space. Pursuit and the LVOR have similar functions: to maintain

images of moving objects on the fovea, be it from self-translation of the body, or movements of an object of interest across the visual world. Hence pursuit and the LVOR may be closely allied, both functionally and phylogenetically. There is already evidence in the literature that the low-frequency LVOR may depend upon an intact pursuit system for its proper elaboration. Accordingly, there may also be a tight relationship – and a consequent possibility for facilitation of learning – between adaptive control of smooth pursuit and of the LVOR. Because of this close relationship, a strong potential exists for developing adaptive paradigms for smooth pursuit that will be applicable to training of the LVOR. Thus, a critical countermeasure for disabling effects of altered gravity on otolith-ocular reflexes might involve training of smooth pursuit eye movements with the head still, both before and during flight.

Along the same lines, context-specific learning – learning to gate in or out a different adapted response depending upon a particular sensory cue – can be studied in the rhesus monkey model. We have evidence for such learning in the intact rhesus monkey using a paradigm of (vertical) orbital-position-dependent learning for the horizontal pursuit response (gain increase in one vertical position, gain decrease in the other). We present here results from a similar procedure on human subjects. The potential arises for training different pursuit gains which in turn may transfer to the LVOR in different gravity environments. It also may be that simply training a subject to have multiple pursuit gains with various contexts in a normal g environment, may make it easier for subjects to adapt their translational LVOR reflexes to different gravity environments. Finally, discovering the contribution of the vestibulocerebellum to pursuit and LVOR learning in general and context-specific learning, in particular, will almost certainly have important practical implications for developing countermeasures based upon ocular motor and vestibular training.

Results – LVOR and the Vestibulocerebellum

We now have important observations on a monkey that underwent bilateral removal of the flocculus and paraflocculus. Immediately post-op, the animal showed signs of a vestibular imbalance with some spontaneous nystagmus (slow phases to the left), a relatively decreased caloric response on the left side, and some asymmetry in the angular VOR. These asymmetries and spontaneous nystagmus resolved within a week. The animal was left with findings typical of a bilateral flocculus/paraflocculus lesion (impaired horizontal pursuit with relative sparing of vertical pursuit, some downbeat nystagmus, and gaze-evoked nystagmus). Rather striking, however, was that after lesioning the animal showed a virtually complete loss of the horizontal LVOR (at 0.7 Hz), during translation both in the light and in the dark, even when the angular VOR in the dark (and the light) had recovered quite well.

These results suggest a critical role for the vestibulocerebellum in the generation of the LVOR (at least at the frequencies measured here), and a tight relationship between the circuits that generate the LVOR and those that generate smooth pursuit. An important caveat here is that the animal also showed some saccade pulse dysmetria, which is not a feature of isolated flocculus/paraflocculus lesions. Whether there was some damage to adjacent cerebellum, the cerebellar peduncles, or brainstem remains to be shown.

Results – Context-Specific Pursuit Adaptation

Figure 6.2 presents the result from a human subject who had undergone pursuit adaptation. (This experiment was also successfully performed with rhesus monkeys.) Shown is the time course of change in initial acceleration from a subject in which two states of horizontal pursuit initiation are called for depending upon vertical eye position. Adaptation is elicited using a paradigm analogous to the double target jump paradigm to elicit saccade adaptation. Using the Rashbass (step-ramp) tracking paradigm the target initially steps away from fixation and then

ramps back toward the initial position so that it crosses the fovea at about 200 ms after target onset (to eliminate saccades). The target then abruptly changes velocity, which serves as the stimulus for pursuit adaptation. Pursuit tracking during training changed in a random fashion between up and down gaze. The left panel in the figure shows the gain decrease paradigm (half pursuit velocity) with the eyes up 5 deg, and the right panel shows the gain increase paradigm (double pursuit velocity) with the eyes down 5 deg. After adaptation, with the eyes up the initial acceleration gradually decreased, with a change during 100 trials based upon the first and last values from the regression line of 14.4 %. With the eyes down, the average acceleration gradually increased, with a change of 47.1%.

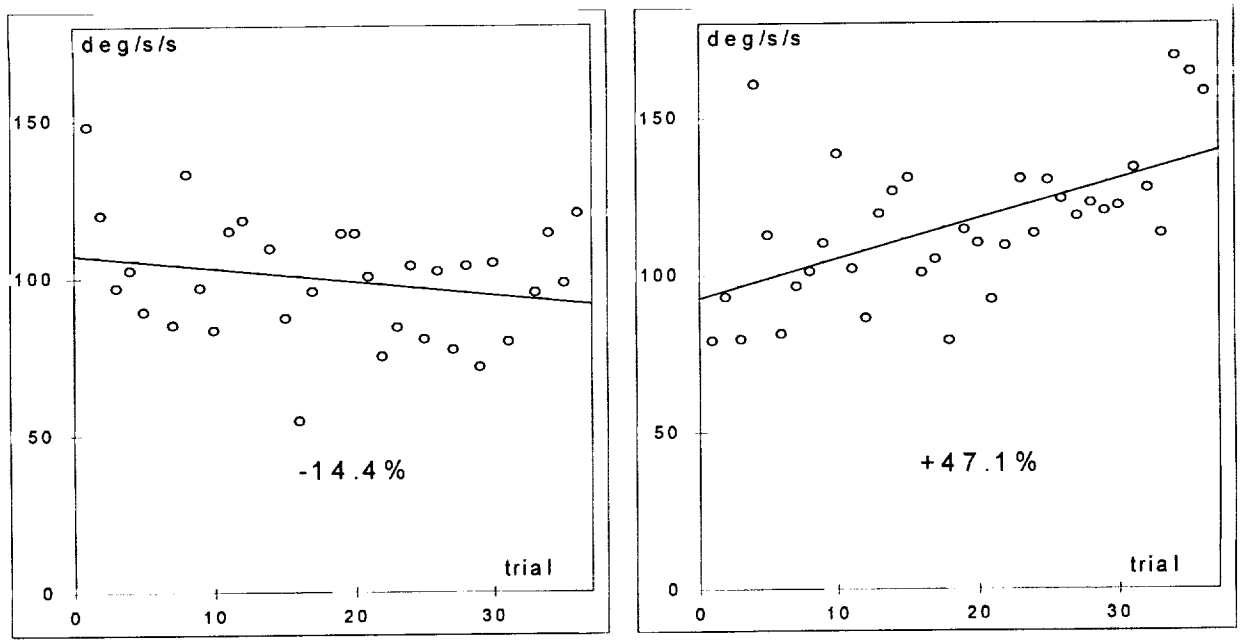


Figure 6.2. Example of context-specific pursuit adaptation. Left panel: gain-decrease (50%) with the eyes up 5 deg. Right panel: gain-increase paradigm (200%) with the eyes down 5 deg. Abscissa is trial number, ordinate is average acceleration during initial 100 ms of pursuit tracking. Dashed lines are regression lines.

Results – Eye Rotation Axis during Pursuit, AVOR, and LVOR

During horizontal eye movements with the eyes raised or lowered, the axis of eye rotation changes in manner that affects the amount of torsional eye motion. We measured horizontal, vertical, and torsional eye movements in four subjects, while horizontal eye movements were evoked in three different ways: smooth pursuit (SP), interaural translational LVOR, and rotation about an earth-vertical axis (AVOR). All stimuli were presented in darkness, and consisted of a sine wave at 0.7 Hz. The LVOR stimulus (translation on a linear sled) had a peak-to-peak displacement of 39 cm (peak velocity 86 cm/s; peak acceleration 0.38 g). SP and LVOR were measured at three viewing distances (43, 100, 200 cm), and the AVOR was measured at 100 cm. Vertical eye positions were -10°, 0°, 10° at the 100 and 200 cm viewing distances, and -20°, 0°, 20° at the 43 cm viewing distance.

The tilt angle of eye velocity about the pitch axis (arctangent of the ratio of torsional to horizontal eye velocity) was fit by least squares linear regression to vertical eye position; the slope of this fit is a measure of the variation of tilt angle with vertical gaze. At 100 cm, slopes were: 0.62±0.06 for LVOR, 0.62±0.08 for SP, and 0.14±0.09 for AVOR. The slope for LVOR

correlated with that for SP ($r^2 = 0.58$, all distances). For LVOR and SP, small differences in tilt angle slope were seen across the range of vergence angles.

These findings provide further evidence for a close relationship between SP and the LVOR, perhaps mediated through shared premotor circuitry. Systems optimized for foveal vision (LVOR, SP, and saccades) seem to use a motor strategy in which torsional eye velocity approximates that predicted by Listing's Law. In contrast, the AVOR may reduce the tilt angle to minimize image slip on the entire retina.

II. Implications of Project Findings for Future Research

Aim 1: Context-specific Adaptation in Parabolic Flight

This experiment can impact the design of countermeasures by showing that a sensorimotor response such as saccades can indeed be made context-specific, with gravity serving as the context cue. It is the only experiment currently in progress to use variations in magnitude of gif as a context cue (other experiments use orientation with respect to gravity as an analog). As noted in section I, a gravity reference may be important for accurate saccade programming. We are considering ways to investigate this further, possibly combining trajectory orientation aspects with context adaptation.

During space flight, there is a change in the static torsional (about the line of sight) positions of the eyes. We have found in other work in our laboratory that vertical errors are introduced in horizontal saccades when the eyes are deviated in torsion. These factors together may result in small but detrimental saccadic dysmetria during flight; the ability to adapt saccades to counter this dysmetria in space, while maintaining the appropriate gravity-based behavior, would be an important countermeasure. To the extent that torsional deviations can cause mislocalization of peripheral retinal cues and other forms of disorientation, our saccade adaptation provides a potential countermeasure for some of the neurovestibular effects of space flight.

Aim 2: Context-specific Adaptation of the Human LVOR

These LVOR adaptation experiments are relevant to the development of countermeasures in that they provide information on the types of vestibular response that might be subject to context-specific adaptation, with gravity serving as the context cue. Since tilt-translation interpretation remains one of the prime candidates for context-specific modification (while recent relevant Neurolab experiments are being evaluated), and since it involves gravity in a direct way, then the use of gravity per se as a context cue may be sufficient for reliable switching between two adapted modes of tilt/translation interpretation.

Our results indicate that head pitch is not an effective cue for the inter-aural LVOR, while head roll is effective. Since roll stimulates the otolith organs in the same manner as the stimulus that elicits the LVOR in this case, our results suggest that the context cue must be closely related to the response being adapted in order to be effective. Future experiments (proposed in our NSBRI renewal) will test this hypothesis by using head pitch and roll as context cues for the LVOR stimulated by fore-aft motion; in this case we would expect pitch to be an effective context cue rather than roll.

One thing we have noticed from several of our past and current experiments is that adaptation is often most effective in "unusual" circumstances. (Although speculative at this point, we think this may be because the "usual" circumstance invokes responses that have been heavily learned through experience, and are thus more difficult to reprogram.) As an example, in our 1993 study of adaptation

of the gain of the AVOR (J Vestib Res, 3:181-195), we found that gain changes were greater if adaptation was induced with the head tilted in roll than if the head was upright. We may be able to take advantage of this phenomenon by using unusual head orientations in our LVOR context experiments. This line of reasoning has implications for countermeasure development because we would be attempting to implement one set of adapted responses in the environment of normal earth gravity, with which the nervous system has lifelong experience, and another set of adapted responses in the unusual environment of space flight.

Another aspect of context-specific adaptation which we will explore is consolidation. Through presentations by and discussions with our JHU colleague Reza Shadmehr, we have become aware of this phenomenon. It is a mechanism by which a learned or adapted response becomes more firmly fixed in its neural substrate, and less subject to destructive interference from other learning (Shadmehr R, Holcomb HH (1997) Neural correlates of motor memory consolidation. Science, 277:821-825). We plan to see if context-specific LVOR adaptation may be enhanced by taking advantage of the consolidation effect. After adaptation to each context for the usual amount of time (currently 5 min), the subject will be allowed to leave the sled and go about normal activities for an hour or so, after which adaptation to the other context will take place for the standard time period, and so on until a total of one hour of adaptation has taken place. With this approach, it is hoped that any interference between contexts might be reduced, since exposure to each context is separated by a period during which the previous adaptation can be consolidated.

Aim 3: Context-specific Adaptation of Oculomotor Responses to Centrifugation

The finding that adaptation does work in principle opens up a large field of new research questions. Not only do we need to find out what method of adaptation is best suited for who and under what circumstances, we also need to identify the context cue for adaptation: what triggers the state change between the normal functioning of the vestibular system and the reinterpretation of its output during centrifugation? For instance, we need to know what resources to allocate to creating an environment that reliably triggers the desired adaptive state. Also, if we would like to pre-adapt astronauts to a cross-coupled environment it may be important to know whether to focus their attention on the internal vestibular signal, on some external context cue, or on both.

Future experiments will also be needed to study generalization of the adaptation. They could involve adapting to the context of CW vs. CCW rotation. In a rotating space station, astronauts can face in opposite direction when standing on the rim, with consequent reversal in the direction of the Coriolis stimulation for a given head movement. This may be a more important daily challenge than a 0 g to artificial-gravity transition, and it is also a more complex adaptation problem because the context involves the direction in which one is facing.

This experiment impacts on the use of artificial gravity as a countermeasure. Head movements on our rotating platform can be designed to produce a variety of signals to the semicircular canals and to the otolith organs, and consequently a variety of new motor commands to the neck muscles and to whole body stabilization can be imposed.

Aims 4 & 5: Properties and Context-specificity of Vestibulocollic Reflex

Our results indicate that the human head-neck system can adaptively reduce head oscillations caused by a drastic change in head inertia, and suggest that the system can maintain adaptation to more than one inertia level. These findings also demonstrate the feasibility of short-term training procedures that would produce long-term preparedness for changes in head stabilization mechanisms. Such procedures would prepare crews of long-term missions for rapid transitions between different gravito-inertial environments by attenuating the effects of these transitions on

head-neck system output. In place of the increased inertia of the current study, other mechanical parameters or feedback can be used for manipulating particular components of the head-neck system and inducing specific adaptations.

It is difficult to infer from available quantitative information any specific effect of spaceflight on the head-neck system or on head stability in general. Changes in head-trunk coordination, accompanied by oscillopsia, have been quantified as reductions in head-trunk coherence after short-duration flight (Bloomberg J, et al. J Vestib Res, 7:161-77, 1997). More recent results confirm these disturbances in three-dimensional head-movement control and demonstrate a concomitant loss of dynamic visual acuity (DVA) after long-duration flight (Bloomberg J, et al. Abstr. of Satellite Meeting of Society for Neural Control of Movement, April 1999). Information that would be directly relevant to head-neck system dynamics in space are not available in the literature. One study illustrated a gradual increase in amplitude of voluntary roll head movements in space after a small initial decrease (Clarke A, et al. J Vestib Res 3(3):207-18, 1993). Amplitudes were abnormally high upon return to earth. This apparent recalibration of sensory-motor programs was considered to be responsible for the quasi-ataxia seen in astronauts in the first few days after landing.

To obtain a more direct indication of changes in head-neck system parameters associated with transitions to and from space, we have applied quantitative methods developed in this project to flight-related data available from other laboratories. A preliminary examination of voluntary head movement data in the pitch axis suggests that head velocity increases over the duration of flight; it becomes significantly larger than its pre-flight value upon landing. This is consistent with Clarke's observation for the roll axis. In contrast to Clarke's study, the faster movements were carried out with visual feedback and landed precisely on target, although they took longer to complete than pre-flight movements. The likely explanation is that the head-neck system oscillated more due to decreased damping in-flight and multiple corrections were required to reach the target. The next stage of our analysis will address these issues. Regardless of the explanation, results to date do indicate severe disturbances in the control of head movements associated with space flight.

Aims 6, 7, 8: Cerebellar Contribution to Context-specific Adaptation in Monkeys

We have now developed a number of paradigms for vestibular learning in animals that will directly bear on issues related to countermeasures. By studying pursuit adaptation we have a way to study its transfer to the LVOR to determine the role of pursuit in the generation of the LVOR, and the potential role of pursuit adaptation alone in eliciting adaptation of the LVOR (a potential powerful countermeasure). Finally, we will be able to study the role of the vestibulocerebellum on these different types of learning in our flocculectomized animals, and to parse out the role of the vestibulocerebellum in the control of the angular and linear VORs.

To a large extent the animal work is basic science aimed at localizing the neuroanatomical site of context-specific learning. Use might be made of this information, however, in the design of human countermeasures procedures, by helping to determine to what extent such behavior is reflexive (learning presumably mediated by the cerebellum) and to what extent it is cognitive (cortically mediated). Adaptation and training strategies should certainly be tailored differently depending on this information. Even in normals, we see a similar range of adaptive strategies, especially with the LVOR in which some subjects use saccadic tracking rather than in increase in the amplitude of the smooth components to increase LVOR gain. Smooth pursuit is also interrupted by saccades in patients with cerebellar disorders. With the lesion experiments, if our hypothesis is true that adaptation is mediated by the cerebellum, then we can determine what

adaptive strategies are used when cerebellar function is disrupted. These same strategies could then be used in humans who exhibit what we might call "non-cerebellar" adaptation strategies – strategies such as saccadic intervention.

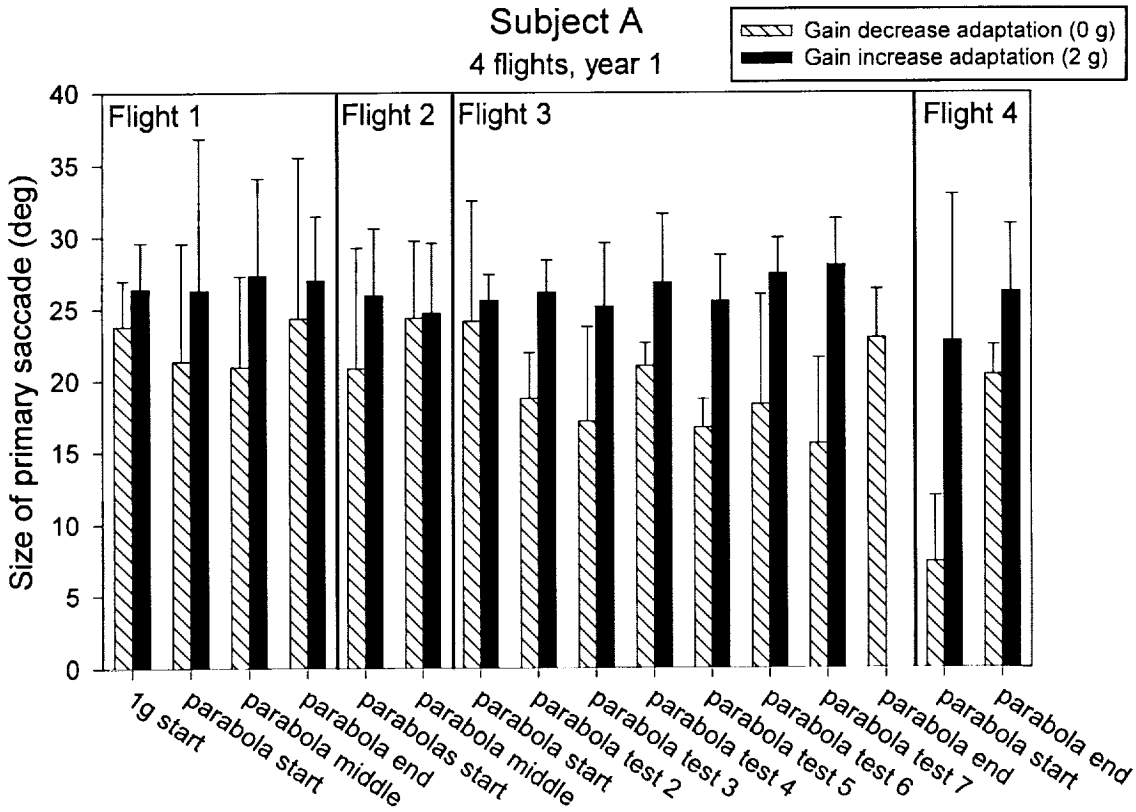
It is critical to elucidate the role of the cerebellum in the various adaptations described here. This will be important in terms of basic knowledge of neural signal processing, but there is a very serious implication for countermeasures as well. If, as seems likely, the cerebellum is adversely affected by some aspects of space flight, then its ability to implement the adaptation-based countermeasures that may be designed based on our results is suspect. We would be remiss to propose countermeasures based on adaptation of vestibular and oculomotor responses without assessing the involvement of the cerebellum, since this issue could make our entire endeavor moot. More specifically, humans and monkeys exposed to altered g environments may show abnormal AVOR and oculomotor function both in 0g and upon return to earth, including spontaneous, gaze-evoked, and rebound nystagmus, impaired pursuit, torsional eye misalignment, incorrectly directed AVOR responses, impaired eye-head tracking, square-wave jerks, and abnormal responses to linear accelerations such as abnormal OCR, positional nystagmus, and abnormal vergence responses during OVAR [reviewed in: Reschke, et al. (1997) Neurosensory and sensory-motor function. In: Huntoon, Antipov, Grigoriev (eds), Space Biology and Medicine. Vol. III. Humans in Spaceflight. Reston VA: AIAA, pp. 135-193]. Some of these abnormalities are enduring and may recur transiently even months after return to earth, suggesting that mechanisms underlying contextual adaptation may be at fault. Many of these findings resemble those reported in cerebellar patients or cerebellar-lesioned animals.

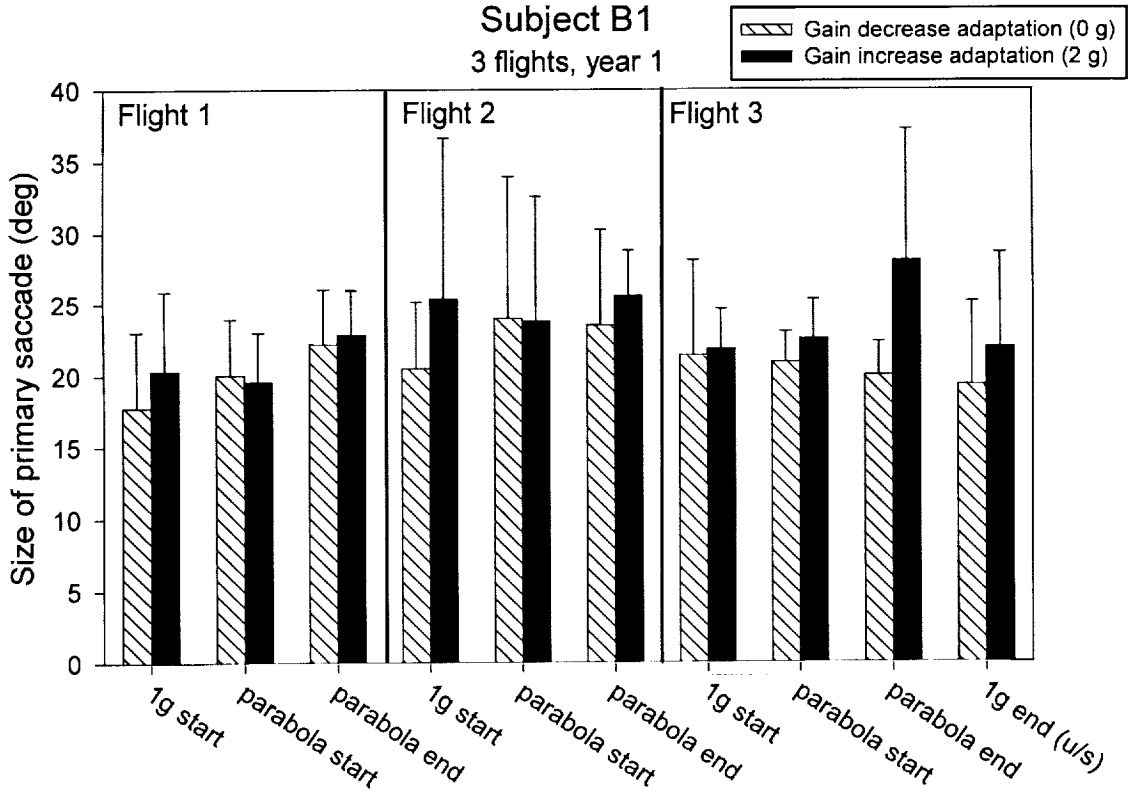
Other fundamental adaptation issues which will be addressed by the animal experiments can also directly impact the design of human countermeasures. Animal experiments can answer some of these questions more readily than can human experiments. For example, one countermeasure often proposed involves adaptation of ocular tilt and translation responses. Tilt is generally a low-frequency phenomenon, and translation high-frequency. Adaptation of otolith-ocular reflexes may be more effective over a certain frequency range, and if so then those frequencies might be more effectively used in adaptation strategies. Investigation of LVOR adaptation over a wide frequency range (especially very high frequencies) is more easily accomplished in animals than in humans. As another example, cerebellar lesions in humans seem to have a large detrimental effect on the low-frequency translational LVOR, while sparing the torsional tilt response. If otolith-ocular adaptation is mediated by the cerebellum, then tilt or translation adaptation can be selectively targeted in humans, depending on any negative effects of zero-g on general cerebellar function (for which anecdotal evidence exists). Since the specific role of the cerebellum in the LVOR is unknown, our animal work is directly pertinent.

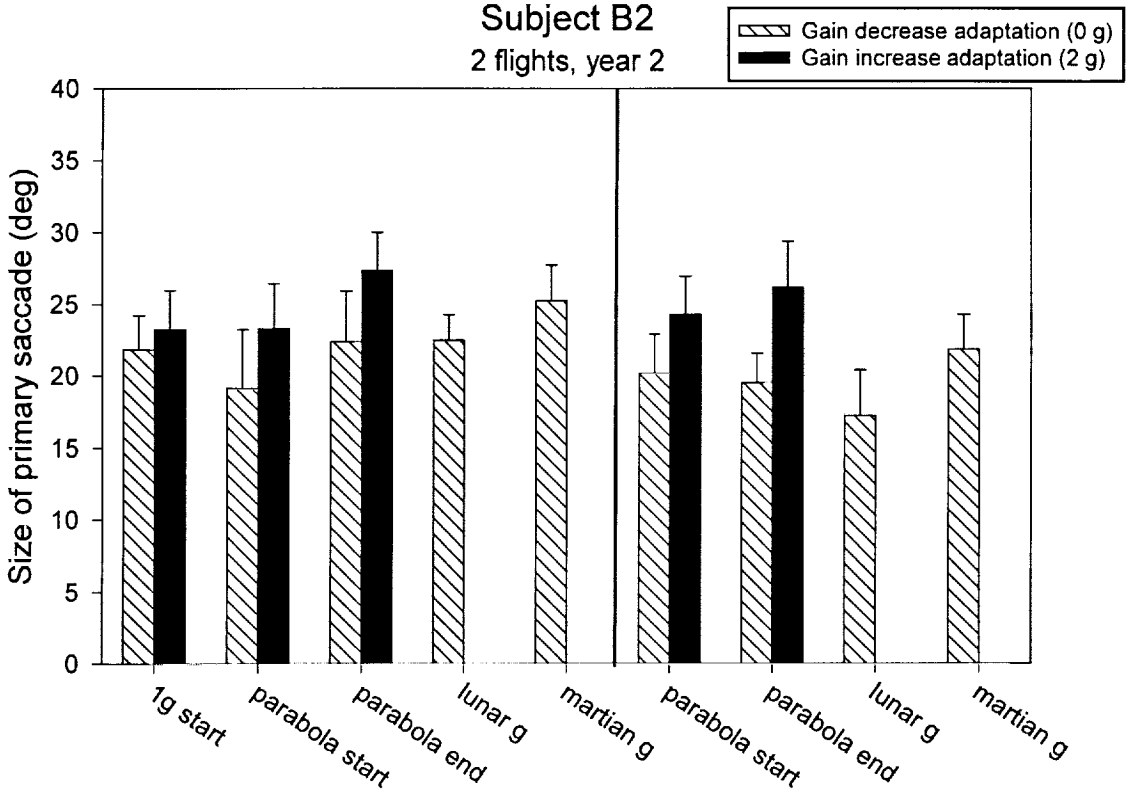
Appendix A

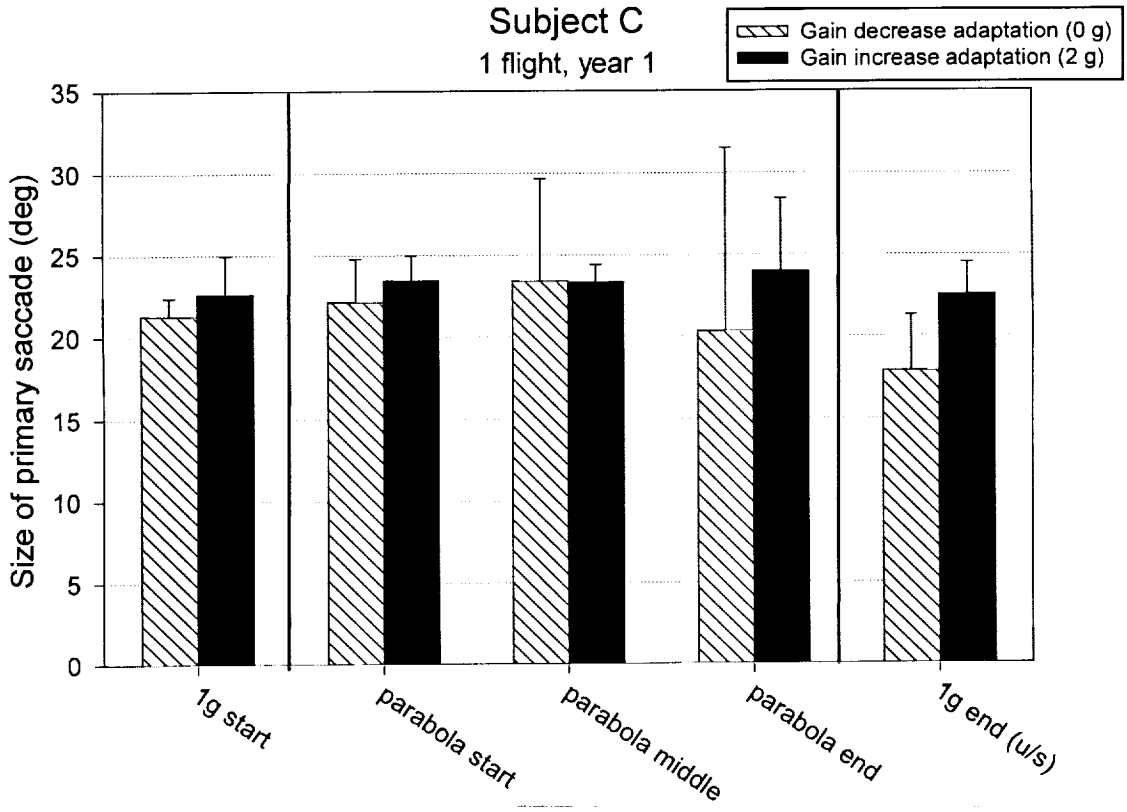
Project Research Data

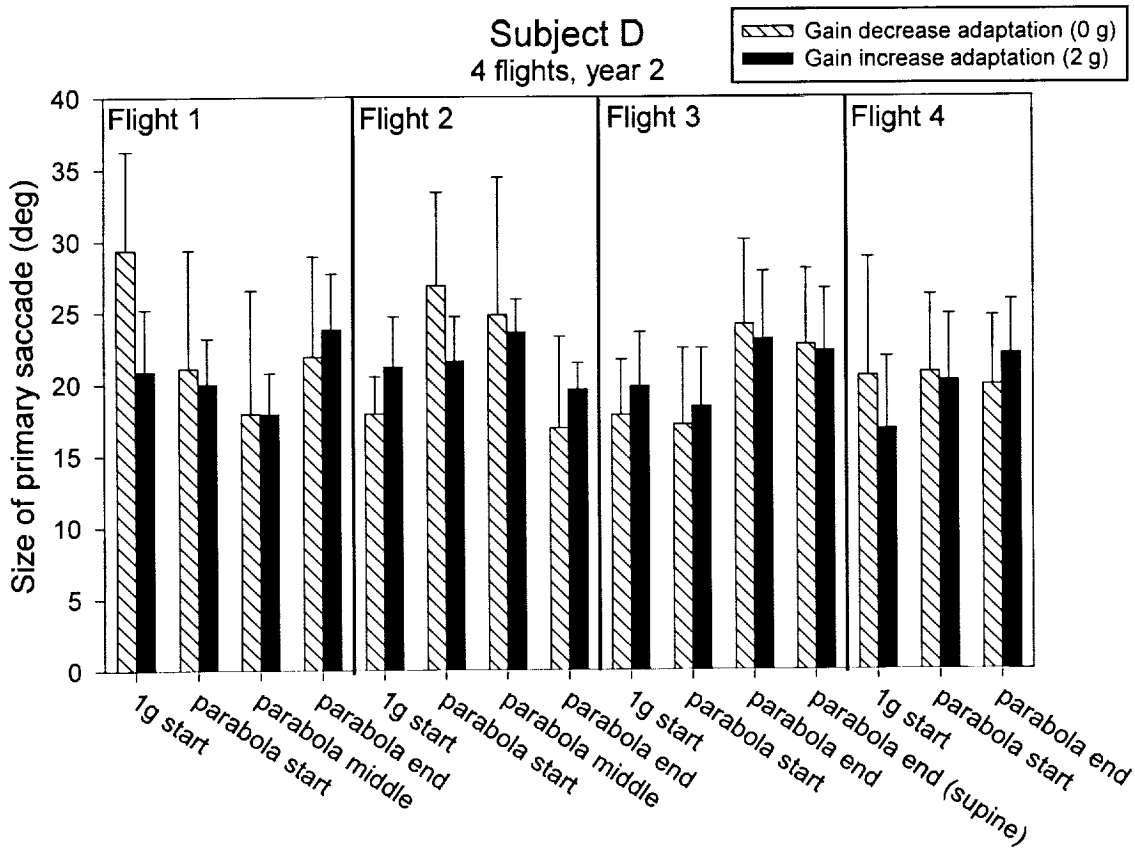
1. Aim 1: Saccade adaptation data from parabolic flight, in bar graph form.

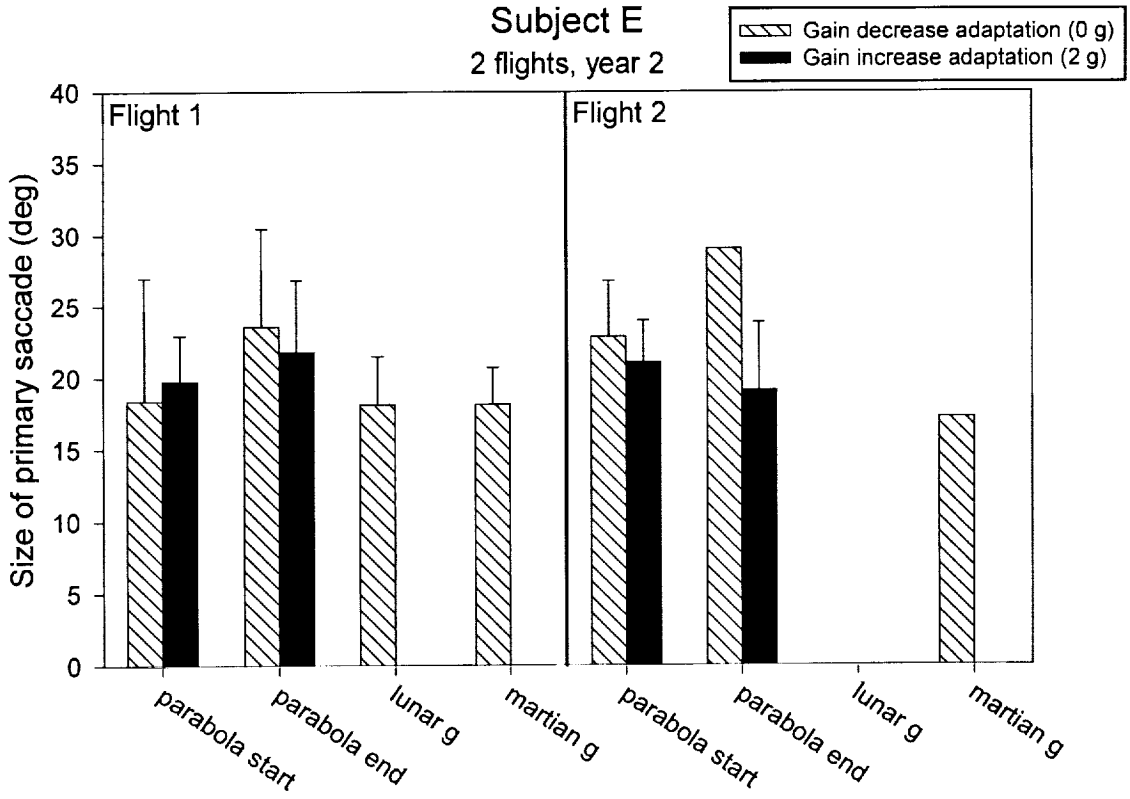

Subject A
4 flights, year 1
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Size of primary saccade (deg)
0
5
10
15
20
25
30
35
40
Flight 1
Flight 2
Flight 3
Flight 4
1g start
parabola start
parabola middle
parabola end
parabolas start
parabola middle
parabola start
parabola test 2
parabola test 3
parabola test 4
parabola test 5
parabola test 6
parabola test 7
parabola end
parabola start
parabola end

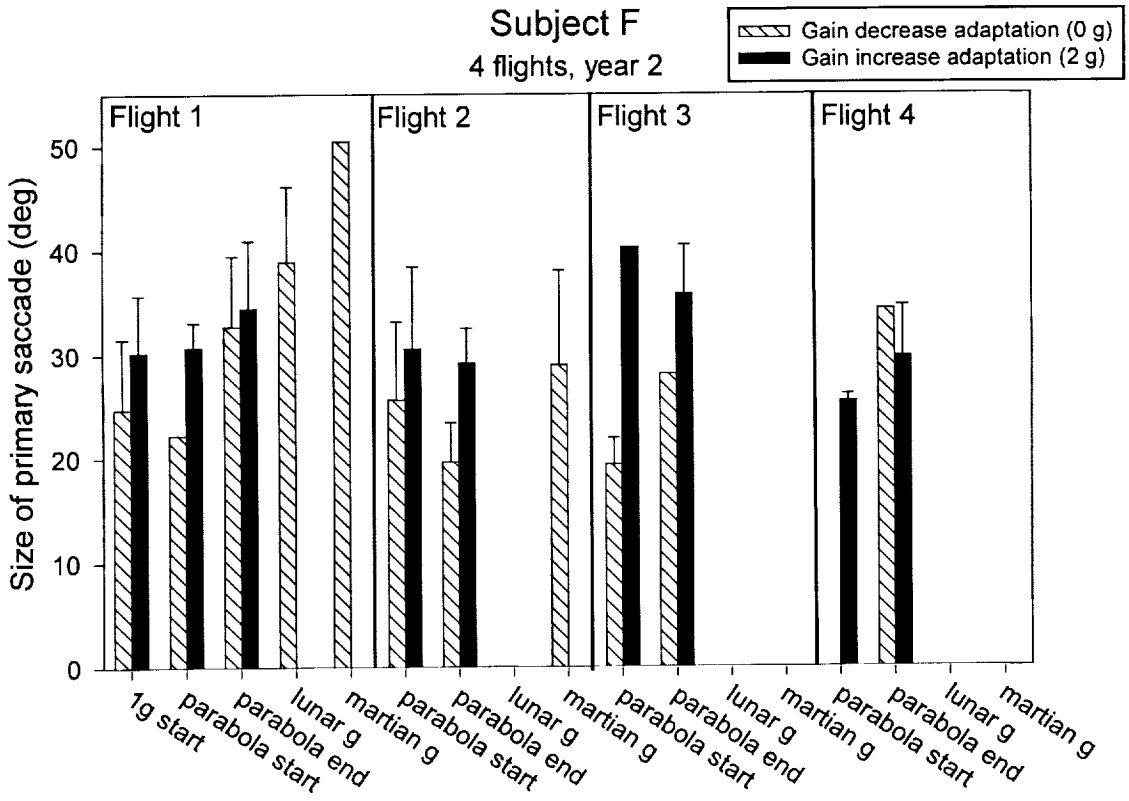

Subject B1
3 flights, year 1
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Flight 1
Flight 2
Flight 3
Size of primary saccade (deg)
0
5
10
15
20
25
30
35
40
1g start
parabola start
parabola end
1g start
parabola start
parabola end
1g start
parabola start
parabola end
1g end (u/s)


Subject B2
2 flights, year 2
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Size of primary saccade (deg)
0
5
10
15
20
25
30
35
40
1g start
parabola start
parabola end
lunar g
martian g
parabola start
parabola end
lunar g
martian g


Subject C
1 flight, year 1
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Size of primary saccade (deg)
35
30
25
20
15
10
5
0
1g start
parabola start
parabola middle
parabola end
1g end (u/s)


Subject D
4 flights, year 2
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Size of primary saccade (deg)
40
35
30
25
20
15
10
5
0
Flight 1
Flight 2
Flight 3
Flight 4
1g start
parabola start
parabola middle
parabola end
1g start
parabola start
parabola middle
parabola end
1g start
parabola start
parabola end
1g start
parabola end (supine)
parabola start
parabola end


Subject E
2 flights, year 2
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Flight 1
Flight 2
Size of primary saccade (deg)
0
5
10
15
20
25
30
35
40
parabola start
parabola end
lunar g
martian g
parabola start
parabola end
lunar g
martian g


Subject F
4 flights, year 2
Gain decrease adaptation (0 g)
Gain increase adaptation (2 g)
Flight 1
Flight 2
Flight 3
Flight 4
Size of primary saccade (deg)
0
10
20
30
40
50
1g start
parabola start
parabola end
lunar g
martian g
parabola start
parabola end
lunar g
martian g
parabola start
parabola end
lunar g
martian g
parabola start
parabola end
lunar g
martian g

Appendix B

List of publications supported through NSBRI funding and appropriately acknowledged

C Cheung (2000) Regulator control of a short-arm centrifuge and subjective responses to head movements in the rotating environment. Masters thesis, Department of Aeronautics and Astronautics, Massachusetts Institute of Technology.

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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

FINAL PROJECT REPORT

Research Team: NEUROVESTIBULAR ADAPTATION

Project Name:

VISUAL ORIENTATION IN UNFAMILIAR GRAVITO-INERTIAL ENVIRONMENTS

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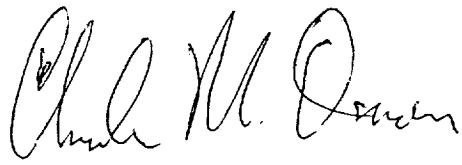
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VISUAL ORIENTATION IN UNFAMILIAR GRAVITO-INERTIAL ENVIRONMENTS

Executive Summary

NASA Life Science's "Critical Path Roadmap" report recognized that the most overt change affecting an astronaut in space flight is the immediate response of the neurovestibular system to changes in gravity level. The large majority of astronauts experience some degree of space motion sickness, inflight disorientation, as well as postlanding sickness, vertigo, and ataxia. Postlanding effects are more severe after long duration missions, which suggests that neurovestibular adaptation to weightlessness – originally thought to be complete after 3-5 days – is in fact a longer process, occurring over a timescale of weeks to months. At first, the neurovestibular problems on the early Mercury, Gemini, and Apollo flights were attributed only to the effects of weightlessness on the inner ear vestibular organs. However, cremembers in the larger Skylab, Spacelab, and MIR vehicles have described disorientation episodes, visual reorientation illusions, spatial memory and navigation difficulties, and EVA acrophobia in which vision clearly plays a major etiologic role. NASA's Critical Path Roadmap defines spatial disorientation and reduced performance on associated cognitive and physical tasks as one of the primary biomedical risks of spaceflight.

How do we know our location, orientation, and motion of our body with respect to the external environment ? On earth, gravity provides a convenient "down" cue. Large body rotations normally occur only in a horizontal plane. In space, the gravitational down cue is absent. When astronauts roll or pitch upside down, they must recognize where things are around them by a process of mental rotation which involves three dimensions, rather than just one. While working in unfamiliar situations they occasionally misinterpret visual cues and experience striking "visual reorientation illusions" (Oman, et al, 1984; 1986, 1988), in which the walls, ceiling, and floors of the spacecraft exchange subjective identities. VRIs cause disorientation, reaching errors, trigger attacks of space motion sickness, and potentially complicate emergency escape. MIR crewmembers report that 3D relationships between modules - particularly those with different visual verticals - are difficult to visualize, and so navigating through the node that connects them is not instinctive. Crew members learn routes, but their apparent lack of survey knowledge is a concern should fire, power loss, or depressurization limit visibility. Anecdotally, experience in mockups, parabolic flight, neutral buoyancy and virtual reality (VR) simulators helps. Unfortunately, our understanding of how our sense of place and orientation is coded in three dimensions in the brain is incomplete.

The role of the NSBRI neurovestibular adaptation team is to do the critical experiments that provide a rationale and methodology for scientifically based countermeasures against inflight and postflight disorientation and motion sickness. The research spans Countermeasures Readiness levels 1-6. Our specific project focuses on the role of visual cues in disorientation. Countermeasures under consideration or active development potentially generic and mission specific preflight visual orientation training using virtual reality and other simulation techniques. Also human factors standards for use of visual polarity and architectural symmetry cues, and the design of escape path signs, allocentric visual landmark systems, and you-are-here maps. Some of these are mature enough so that during the next year, we plan to begin working with the NASA JSC Countermeasures Evaluation and Validation Project to initiate non-advocate, evidence based review and formal validation and implementation of some of our concepts. Meanwhile, a member of our research team (J. Richards) is spending 3 months at JSC this fall, working with Dr. Jon Clark, a flight surgeon who leads the JSC Neurological Function Integrated Project Team, to compile a more detailed and quantitative record concerning the actual operational incidence of visual orientation problems in the NASA-MIR and ISS programs.

The three major research themes (specific aims) of our project and the principal findings are:

1) Human visual orientation. (I. Howard, et al, York University). We have studied how visual cues determine spatial orientation and how ambiguous cues cause visual reorientation illusions. Using an 8 foot "tumbling room" at York University, whose interior was furnished with tables, chairs, bookshelves, etc. we investigated the conditions in which the perception of self orientation with respect to the vertical is dominated by gravity, the visual frame of reference provided by the room's realistic interior, or by the principal axis of the subject's body. There is a natural tendency to perceive the feet as "down". It has long been known that moving visual scenes can produce compelling illusions of self motion (e.g. flight simulators and wide screen movie theaters), but it was not understood that motionless visual scenes could produce very large sensations of static tilt under certain circumstances. Howard and Hu (2000) showed that when gravitationally supine subjects viewed the interior of the furnished room that was similarly tilted 90 degrees with respect to gravity, so that it appeared upright with respect to their body, a majority of subjects reported they felt gravitationally upright. We call this a "Levitation Illusion". If subjects extended their limbs above their supine body, their limbs felt weightless. The strength of the illusion has been systematically studied in a large group of subjects with the room and the subject in all the different possible orientations, modulo 90 degrees. In certain other relative orientations, subjects experienced visual reorientation illusions – for example they perceived the floor of the room as a ceiling. We were surprised to see that susceptibility to the levitation illusion consistently increased with the age of the subject. Vestibular function is known to degrade with age, and the association between the orientation of familiar visual objects and gravity (which we refer to as "visual polarity") is probably a learned phenomenon. In a related experiment, we also constructed a novel "mirror bed" device, which allowed us to quantify how the object property we refer to as "visual polarity" determines the strength of a VRI. A subject lying gravitationally supine in the bed views the laboratory through a mirror mounted at 45 degrees over his head. When strongly polarized objects are in view, the subject interprets the view as horizontal, and feels subjectively almost upright. The sensation of tilt is sufficiently compelling to produce visual-autonomic responses (Wood, et al 2000). When weakly polarized objects are seen, the subject feels nearly supine. Intermediate tilt perceptions can be created by manipulating the polarity (type and arrangement) of objects in the visual scene. Some objects (e.g. desks, chairs, saucers) have "intrinsic" polarity, because in daily life they are consistently seen in a specific orientation with respect to gravity. Other objects (e.g. blocks, pens, books) seem to have no intrinsic polarity until they are placed "on" another object. In this case, through their physical relationship the pair acquire what we refer to as "extrinsic" polarity. Understanding how the relative orientation of gravity, body axis and the visual scene interact is potentially very important for astronaut training, and also in entertainment and clinical applications. Strongly polarized objects and pictures may prove useful in reducing the incidence of disorienting VRIs in space station modules. Placing strongly polarized pictures in staircases might help some elderly people be less prone to falling.

2) Three dimensional spatial memory and learning. (C. Oman, et al, MIT and W. Shebilske, Wright State Univ.) On Earth, humans have a remarkable ability to keep track of their orientation and position relative to local landmarks. However, most large body rotations are made about the body axis which is aligned with the gravitational vertical. What are the limits of human ability to imagine, orient and navigate in a weightless environment, where one is free to turn completely upside down ? MIR astronauts reported great difficulty visualizing the relative orientation of different modules on the station, and remaining oriented when traversing the central node module. Similar problems are anticipated on the ISS. Can spatial abilities in such 3D environments be improved by preflight training ? Most navigation and spatial memory research has addressed only the terrestrial situation. To find out, we have conducted a series of four experiments on human spatial memory. We designed a 3D spatial task (Oman, et al, 1999, 2000; Shebilske et al, 2000; Richards, 2000; Richards et al, in preparation) analogous to that confronting astronauts trying to learn the spatial relationships between the six entrance hatches in a space station node module of a space station. Subjects were placed in the center of a small six sided room. A picture of an easily recognized and remembered object was located on the center of each wall. Subjects had to memorize the relationship between the pictures, such that they could predict which picture would be where, even after the room was rotated about them into any

one of 24 possible relative orientations. Subjects would be told their orientation relative to one of the walls, or shown the pictures on two of the walls, and then in darkness indicate the relative direction to a specific unseen picture. All six pictures would appear, and the subjects would have a few moments to study the relationships between the pictures before the next trial began. Some constraints were put on relative room orientation during the first two dozen trials to facilitate initial learning. However, by the time the subjects had completed sixty trials, most were able to reliably predict the direction of a specific target picture from any arbitrary orientation. The experiment was conducted using a head mounted display system to render a virtual room. However, it was repeated using a physical room, and very similar results were obtained. Tests also showed the gravitational orientation of the subject had little effect on the subject's ability to perform the task. However, the ability to do two and three dimensional mental figure rotations and recognize imbedded figures, as measured using conventional paper-and-pencil forms similar to those found on IQ tests, did consistently correlate with performance in each of our studies where we have tested it. Exit questionaires suggested our subjects chose to remember the relationships amongst the figures as they would appear with the room in a specific "baseline" orientation – often the orientation they encountered it at the beginning of the test. Many discovered they could memorize opposite pairs of objects, and learn the relationships between groups of three objects, from which the relative direction of all six could be inferred. Prior explanation and practice with the baseline orientation, pairs, and triads concepts tended to improve performance as compared to a control group, but we believe many of the control group subjects discovered these or similar techniques on their own. The best performers were the subjects with strong 2&3D mental rotation scores. Many of this group said they were able to visualize the room interior, and mentally rotate themselves or the room, and make the correct judgement, only occasionally falling back on mnemonic rules like pairs and triads. To see whether subjects "learned how to learn" the task, in some of the experiments we trained subjects in two successive environments. As we hoped, they usually learned significantly faster in the second. In one experiment we brought subjects back in for retesting and found ability was retained one day, one week, and even one month after initial training. Another experiment showed that learning with randomly chosen rather than grouped (blocked) sets of room orientations enhanced ultimate performance. We are currently working on extending the paradigm to measure spatial memory across two previously learned modules, one of which is unseen. We want to know if coalignment of the baseline memorized module orientations is critical for performance. MIR crewmembers reported great difficulty visualizing the relative orientation of adjacent modules when the baseline orientations (established by equipment arrangements and visual verticals) was not coaligned. Our ultimate objective is to develop a methodology/pedagogy for generic and mission specific ISS preflight visual orientation training. Another application of this paradigm is in the design and evaluation of emergency escape route markings and systems of visual landmarks within modules that help crewmembers keep track of the principal axes of the ISS.

3) Neural coding of spatial orientation in an animal model. (J. Taube, et al, Dartmouth) Using a rat animal model, we have conducted experiments in our ground laboratories and in parabolic flight to better understand how our sense of place and direction is coded in 3 dimensions. In rats and primates, "head direction" cells have been found in the limbic system that appear to code head direction in a gravitational horizontal plane, independent of the animal's location, and roll or pitch of the head up to 90 degrees. The direction of maximum response ("preferred direction") lies in a fixed direction which varies from cell to cell. Under 1-G conditions, moving a prominent visual landmark around the animal results in a corresponding re-orientation of the preferred directions of all HD cells by the corresponding angle, a phenomenon that corresponds to the familiar human experience of emerging onto the street level from a subway, and reorienting your sense of direction based on viewing a familiar landmark. Until this project began, the response of HD cells had been studied only in a gravitationally horzontal plane. We have conducted a series of 1-g laboratory experiments in which rats are trained to crawl up a wall, across a ceiling (hanging upside down) and down the opposite wall, in an apparatus that allows us to verify the 3D response characteristics of HD cells, and infer whether the response sensitivity remains anchored by gravity to a horizontal plane, or whether the response coordinate frame of the cell re-orients to the plane on which the animal is locomoting. Results of these 1-g experiments (Taube, et al, 2000) indicate that cells in some animals continue to show robust direction specific firing in the same world-centered reference frame when the animal is walking upside down,

and response on the walls depends on the wall and whether the animal was going up or coming down, as expected. In other animals, the cells lose their direction specific firing on the ceiling. We suspect that some animals may be better at remaining oriented on the ceiling than others. In a separate series of experiments (Taube, 1999 and in preparation), we have also studied HD cell response chararacteristics in parabolic flight in a test chamber that was visually symmetrical in an up-down direction. All cells HD cells studied maintained their direction specific discharge when the animal was on the floor or the wall of the chamber. However, when placed on the ceiling of the chamber, HD cell directional specificity was frequently lost. However, in some cases, the preferred direction of HD cell response reversed across the visual axis of symmetry of the cage, as it would be expected to do if the cell's response coordinate frame reoriented to the ceiling. When humans roll inverted in parabolic flight and put their feet on the ceiling of the aircraft, they experience a visual reorientation illusion in which the ceiling seems like a "floor", and the left-right axis is reversed. We believe this is the first demonstration of the limbic correlate of a human 0-G spatial orientation illusion. Corroborating evidence has also recently come from space shuttle Neurolab experiments on limbic "place" cells which we believe are driven by HD cells (Knerim, et al 2000). Our results have suggested several important physiological questions, such as what mechanisms cause HD cells to lose their directional sensitivity ? What kind of disoriented behaviors does it produce ? Our experiments provide important insights on the role played by gravireceptors in stabilizing the human sense of place and direction not only in astronauts, but also in vestibular and Alzheimer's disease patients. Funding from NASA has allowed us to pursue the anchoring role of gravity as a major theme, and provided access to the unique facilities required for parabolic flight experiments. Involvement in the design of the experiments has helped all members of our project team form appreciate the close relationships between cognitive and cellular events.

It is never possible to predict the direction that an aggressive research program will take, but in retrospect we met or significantly surpassed our original goals in most areas, as defined in our 1997 proposal. Our discovery of 90 degree static 1-G visual reorientation illusions and their strong age dependency, the development of the mirror bed VRI technique, and its use as a research tool to quantify the strength of visual polarity cues and for vestibular autonomic research all were not anticipated. At the suggestion of advisory committees, our work on orientation and navigation has focussed on development and validation of our "virtual node" experimental paradigm, and comparison with a physical node. The physical node work was not originally proposed. However this focus has now led us to specific concepts for generic 3D spatial memory training, and positioned us well for further research on 3D navigation, and for evaluation of specific signs and landmarks. We have demonstrated a strong correlation between performance in our 3D spatial memory task and measures of mental rotation ability, and not just field dependence, as we originally proposed. Our study of the effects of foot pressure cues is still in progress. We have not attempted to assess the effects of an individual's "gravireceptor bias", as originally proposed, nor have we studied postural responses in response to scene movements. The latter study was to be part of a collaboration with another project which was not funded. Our development of an animal model for visual reorientation illusions in 1-g and 0-g has ultimately been successful, although it has proven more difficult than first anticipated to train the animals to crawl upside down across a gridded ceiling, so some of this work is still in progress. Another measure of our success is that there have been significant collaborations between investigators at the principal research sites and investigators on other teams. Dr. Oman participated in the design and performance of Dr. Taube's experiements; Dr. Shebilske designed the original "node" paradigm used by the MIT investigators. Dr. Howard and Dr. Oman have collaborated on the design of the VRI experiments, and Dr. Howard's mirror bed technique has been employed by Drs Ramsdell and Wood of the cardiovascular team in their study of transient cardio-respiratory responses to visually induced tilt.

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1. Project Research Activity

1.1 Background

Our goal of this project is to better understand the process of spatial orientation, navigation, and spatial memory in unfamiliar gravito-inertial environments, and to use this new information to develop effective countermeasures against the disorientation and motion sickness problems experienced by astronauts. During the first grant period, our research addressed three themes: 1) human visual orientation, 2) 3D spatial memory and learning, and 3) neural coding of spatial orientation in an animal model.
Since some readers may be less familiar with the physiology and psychophysics of visual orientation, this section provides some background and presents the rationale for our project. Readers acquainted with this may skip directly to section 2, which summarizes our results.

1.1.1 Spatial Orientation on Earth. In daily life on earth, how do we know our location, orientation, and motion of our body with respect to the external environment ? Visual and "idiothetic" (vestibular, haptic, motor efference copy) cues all play important roles when the body is moving. In static situations, the vestibular semicircular canals do not respond, and the otoliths and other gravireceptors provide only a "down" cue, so visual information is usually essential in order to maintain an absolute sense of azimuthal direction. Our sense of static tilt with respect to the gravitational vertical results from the synthesis of available gravireceptor and visual cues, as demonstrated by Witkin et al's (1948) classic tilted room and rod-and-frame experiments. Mittelstaedt (1982) suggested that perceived tilt also depends on an individual's "idiotropic" tendency to align the subjective vertical with the body axis. When a visual scene is present, at least four characteristics of the visual scene are critically important (Howard, et al 1999): 1) Orientation of axes of symmetry of stationary objects and large plain surfaces whose subjective identity depends on viewing aspect. We refer to this as the "frame" effect, since even a tilted square in a dark surround induces self tilt. 2) Intrinsic polarity of certain familiar objects, such as desks, trees, and people, which are consistently oriented with respect to gravity. Typically such objects are bilaterally or radially symmetrical, and have identifiable principal axis with one end perceptually the "top" and the other end perceptually the "bottom". Certain large recognizable surfaces also fall into this group, such as a lawn, water, or the earth viewed from orbit. 3) Extrinsic polarity, defined by relationship of one to another object and to gravity. Examples include objects on shelves, and hanging or falling objects; tapered objects that would fall over unless they were large end "down"; and shaded objects, since light is presumed to come from "above" (Howard, et al, 1990). 4) Scene motion. Even a rotating scene lacking frame and polarity cues induces self-tilt (Held, et al, 1975). The tilt illusion is stronger when the head is tilted away from the vertical or upside down (Young, et al, 1975; Howard et al, 1988).

1.1.2 Spatial Orientation in Weightlessness. In the cabin of an orbiting spacecraft, gravireceptor cues are absent, and the notion of a "gravitational down" becomes meaningless. However, in order to know which way to look or reach for remembered objects, crewmembers must identify objects and surfaces inside the spacecraft, and maintain an exocentric frame of reference while moving about. Astronauts usually speak of the "visual down" reference provided by the interior architecture of the spacecraft. When they float with their feet towards the spacecraft "floor" (as defined by experience in 1-G simulators), crewmembers rarely feel disoriented. However, Skylab (Cooper, 1984) and Shuttle astronauts (Oman et al, 1986) have reported that when working upside down (relative to their normal 1-G orientation in training), or when right side up but viewing another crewmember floating upside down, they frequently experience a sudden change in the direction of the perceived vertical, called a "visual reorientation illusion" (VRI) (Oman et al 1986, Oman et al 1988). In this striking illusion, the surrounding walls, ceiling, and floors seem to exchange subjective identities. For example, crewmembers who face the ceiling or floor sometimes feel "upright". Spacewalking astronauts working upside down in the Shuttle Payload Bay have described looking up at the earth, experiencing a VRI, and suddenly fearing they would fall "down" to Earth. The sudden change in perceived orientation (without concomitant vestibular motion cue) can trigger attacks of space sickness, cause reaching errors, and can make it more difficult for crewmembers to recognize landmark objects. VRIs often occur spontaneously, but are labile, and can be cognitively manipulated. VRIs happen because of the idiotropic tendency to assume the surface beneath one's own feet is a floor,

and because other people are normally seen in a gravitationally upright position. Headward fluid shift and gravireceptor unweighting are believed also to contribute, and make some astronauts feel continuously inverted ("inversion illusion") (Gazenko, 1964; Matsnev et al 1983; Oman et al, 1986; Lackner, 1992). We found significant differences between crewmembers in VRI susceptibility on the recent 19 day Neurolab shuttle flight when virtual spacecraft interior scenes were presented at various tilt angles, using a virtual reality display (Oman et al, 2000) VRIs can also occur on Earth, such as when we exit the subway, and discover we are facing in an unexpected direction. They normally occur in 1-G only about the gravitational vertical, but we have also created them experimentally about the gravitational horizontal using real and virtual tumbling rooms (Howard and Childerson, 1994; Oman and Skwersky, 1997; see also Section 1.2.1). VRIs are analogous to figure reversal illusions (e.g. Necker cube), except that it is the person's own subjective orientation which changes, rather than the subjective orientation or identity of the object.

1.1.3 Spatial Memory and Navigation on Earth. On Earth, humans have a remarkable ability to keep track of their orientation and position via a combination of path integration and visual landmark recognition. Understanding of adult navigation and spatial memory has been influenced by studies of the development of these skills in children (Piaget and Inhelder, 1967; Siegel and White, 1975). When people experience a novel environment, they first learn to identify landmarks. With experience, adults and older children associate individual landmarks with specific actions, such as turning left or right, and eventually learn the sequence of landmarks and actions as a route. Simple route knowledge is based on declarative rules (Anderson, 1982), but with practice, eventually becomes automatic. Most older children and adults recognize common landmarks on interconnected routes. They eventually develop survey (configurational) knowledge of an environment, as evidenced by an ability to take shortcuts, point home, or to describe the environment as it would be seen from a different viewpoint. There is doubt that the landmark/route/survey stages are necessarily sequential rather than concurrent, but the basic model is widely accepted (McDonald and Pellegrino, 1993). Route and survey knowledge can be acquired by direct experience in the environment ("primary knowledge") or learned through map study ("secondary knowledge"). Often people use both techniques. Primary survey knowledge of large scale environments is detailed and relatively automatic. Secondary survey knowledge is thought to involve mental rotation of a cognitive map, making it harder to retrieve. Survey knowledge can involve processes of mental imagery (e.g. Reiser, 1989) that may activate the same brain structures as in direct visual perception (e.g. Kosslyn et al 1993), but there is also evidence that spatial information is hierarchically and categorically organized and processed: Sadalla et al, (1980) found that humans use spatial "reference points" to define the position of adjacent places. Tversky and colleagues (Franklin and Tversky; 1990; Bryant and Tversky, 1999) argue that spatial mental models are based on conceptions rather than perceptions, and that people imagine object locations using a "spatial framework" employing their body axes to establish referent categorical directions. Blind pedestrians appear to use interconnected, modular spatial frameworks (Hollyfield & Foulke, 1983). Klatzky et al (1999) showed that in the absence of vision, humans keep track of where they are by retrospectively judging leg lengths and turns.

Most navigation and spatial memory research has addressed the terrestrial situation, where subjects remain gravitationally upright, and move about on 2 dimensional surfaces. Franklin and Tversky (1990) have noted that mental transformations normally required to imagine a scene from a new perspective normally only involve rotations about a gravitational axis. We think the terrestrial literature provides a useful starting point for research on navigation in 0-G. What are the limits of human ability to imagine, orient and navigate in three dimensions in situations where the subject is free to turn completely upside down ? When we physically change positions in a 1-G environment, the cognitive process of relating spatial relationships in multiple visual perspectives is automatic in the sense of demanding few, if any, cognitive resources, and it is often incidental and implicit (Pick, 1993). These automatic cognitive processes are likely to fail in space for many reasons including novel perspectives, reduced visual polarity information, and altered stimulus-response contingencies in vestibular and proprioceptive systems. People typically have some practice utilizing deliberate, explicit, executive control processes to mentally transform their spatial relations to target objects when reading maps, when struggling to find their way in a new territory, and in some piloting situations. The skills developed in these acute situations are unlikely to be adequate, however, for the astronaut's chronic problem of maintaining situation awareness in conditions that require them to mentally transform their spatial relations to target objects in order to continually update their

mental representation of spatial relationships. Spatial mental models can be considered as a subset of more general ones used to maintain overall situation awareness (Endsley, 1995; Shebilske, Goettl, and Garland, in press).

1.1.4 Spatial Memory and Navigation in Weightlessness. Over the past several years, operationally significant problems involving three dimensional spatial memory and navigation have been anecdotally reported by US and Russian crewmembers. NSBRI Neurovestibular Adaptation team members interviewed four US Astronauts who lived for months on the Russian MIR space station, and several Shuttle crewmembers who visited the station for several days. The MIR research modules connect at 90 degree angles to a central, 6 ported spherical "node". MIR modules have a rectangular interior cross section, and most have brown colored floors, blue ceilings, and tan walls. Unfortunately, the visual verticals of the different modules are not co-aligned. Crewmembers said that even though they knew the physical arrangement of the modules, they could not completely mentally visualize the arrangement. For example, they could not point in the direction of familiar interior landmarks in other modules the way they could when in their homes on Earth. When moving between modules they eventually learned to use landmarks and rules to navigate through the node. One crewmember recalled: "I learned that to go into Priroda, I needed to leave the base block upright, go through the hatch to my left, and then immediately roll upside down so that Priroda would be right side up". Another said: "Even though you knew the modules went in six different directions, it felt like the node was a vestibule in a single story house.... You eventually just learned what to look for and do to get to your destination." A third said: "After I first boarded MIR, I decided to go back to the Shuttle, but discovered I didn't know which way to go, since I hadn't left behind any bread crumbs!". Similar reports have also been made by a fifth US astronaut (Linenger, 2000) and by many Russian cosmonauts (I. Kozlovskaya, personal communication). To assist Shuttle visitors, MIR crew fashioned red velcro arrows, and positioned them on the walls pointing toward the Shuttle adapter hatch. Comparable problems were not encountered within the US Shuttle, probably because the flight deck, mid-deck, and payload bay research modules have coaligned and less ambiguous internal visual verticals. In 1997, several operational crises which occurred aboard MIR convinced crewmembers that the ability to make three dimensional spatial judgements is important in emergency situations and critical if an emergency evacuation is necessary in darkness, or when smoke obscures the cabin. Twice when collisions with Progress spacecraft were imminent, crewmembers moved from module to module and window to window, unsuccessfully trying to locate the inbound spacecraft. Another emergency required the crew to reorient the entire station using thrusters on a docked Soyuz spacecraft. Members of the crew in the MIR base block module discovered they had great difficulty mentally visualizing the orientation of another crewmember in the differently oriented Soyuz cockpit, and verbally relaying the appropriate commands (Burrough, 1998).

Similar difficulties are being encountered on the new International Space Station. NASA human factors standards (e.g. 3000/8.4.3) require locally consistent internal visual verticals. However, as built, the US nodes and modules have a square interior cross section and similarly colored equipment and stowage drawers on both walls, the overhead, and the deck, resulting in ambiguous visual verticals (dual visual polarity, ambiguous frame effects; see below). Six 6-ported node modules are eventually planned. Six modules (Node 3, Hab module, and 4 Russian research modules) will have visual verticals oriented 45°, 90°, or 180° from those in seven other modules) On the first mission to the Station, crewmembers noted it was easier to become disoriented in the US "Unity" node module, as compared to the Russian Zarya control module. The latter has a rectangular cross section, and different colored internal surfaces. The crew placed emergency "Exit" signs beside the node hatches, but subsequently discovered that one the signs had been misplaced, probably as a result of a visual reorientation illusion. Maintaining spatial orientation during EVA activity on the outside of the station was difficult, particularly during the dark half of each orbit, due to the lack of easily recognizable visual landmarks (D. Barry, personal communication).

1.1.5 Previous Visual Orientation and Spatial Memory Countermeasures. The need for preflight visual orientation practice was recognized after Apollo and Skylab. Experience in mockups and parabolic flight is believed to help. Crews routinely practice Extra Vehicular Activity (EVA) in neutral buoyancy facilities. Since 1993, Shuttle crews have used virtual reality techniques for EVA rehearsal. In JSC's Integrated EVA/RMS VR Simulation Facility pairs of astronauts wear tracker equipped stereo head mounted displays (HMDs), electromagnetic forearm trackers and sensor gloves. They maneuver hand over

hand, ride the shuttle manipulator arm, or use a simulated jetpack. The highly detailed visual environment includes the vehicle and a view of the earth. The latter cannot be simulated in water tanks. Crewmembers say the practice they get visually orienting to the Shuttle Payload Bay or the MIR Station is extremely valuable (J. Hoffman, personal communication). This facility has not yet been used for Intra Vehicular Activity (IVA) training.

In the early '90s, Parker and Harm pioneered the development of formal preflight orientation training using the DOME, a simulator which projected visual scenes on a quarter of the interior of a 12 ft. sphere. Subjects controlled their virtual position and orientation with a joystick. The DOME was part of a Preflight Adaptation Training (PAT) research effort whose main goal was preinduction of otolith tilt-translation reinterpretation, and development of a laptop computer based method for reporting self-orientation. A group of astronauts also used the DOME to practice moving about a Spacelab interior while in simulated agravic body orientations. However, the effectiveness of this training on orientation performance was not measured. A retrospective study (Parker and Harm, 1993) suggests that PAT trained astronauts may have had a lower incidence of space sickness. The DOME simulator is not currently operational. Its restricted field of view makes it inappropriate for our proposed research.

It has occasionally been suggested that astronauts be provided with spatial orientation prostheses, analogous to artificial horizons and moving map displays used by pilots (Baldwin, et al 1997, p. E-24). For example, a vibro-tactile vest under development by the US Navy which provides orientation or direction cues to pilots and divers. Head mounted display technology developed for wearable computing applications (e.g. Microvision, Inc. retinal display; Sony Glasstron) could also potentially be employed. Such concepts are appealing, but the costs of space suit modifications necessary for EVA are extraordinary. Even when working inside a vehicle, crewmembers are reluctant to be continuously encumbered by special purpose equipment. Also, a practical system requires an as-yet-unavailable head tracking technology, capable of operation wherever the crewmember goes. (Installation of optical or acoustic trackers in all locations is impractical. Inertial tracking works for short periods only, since gravity and magnetic direction references are not available in orbit, and GPS is unreceivable inside the spacecraft.) We believe that such technology-based spatial orientation aids may be useful in aircraft cockpits, and some clinical situations, including postflight rehabilitation of astronauts. We believe virtual reality displays have a potentially important role in preflight training of astronauts, as detailed later. However we have largely focussed on cognitive training and low-technology solutions (e.g. providing additional visual landmarks) as the most practical and versatile solutions for the spatial disorientation and spatial memory problems astronauts routinely encounter in orbit.

1. 1.6 Neural coding of place and direction. Studies show that animals construct an internal spatial representation of their environment (cognitive map) and use this for spatial navigation (O'Keefe and Nadel, 1978; Mittelstaedt and Mittelstaedt, 1980; Collett et al, 1986; McNaughton et al, 1995). Some of this neural circuitry involves the limbic system and in particular the hippocampus. Although the precise functions of the hippocampus are still debated, there is little doubt that it is essential for processing exocentric (allocentric) spatial information processing, since both animals and humans with hippocampal damage are impaired on a variety of spatial and navigational tasks (Olton et al, 1979; Morris et al, 1982; Landis et al, 1986; Habib & Sirigu, 1987). Additional evidence comes from studies showing that two types of spatial cells in the limbic system code exocentric spatial information. "Place cells" have a response component related to the animal's location in the environment. Place cells have been reported in the rodent (O'Keefe, 1976; Muller, 1996) and comparable spatial view cells have been identified in the primate hippocampus (Rolls & O'Mara, 1995). "Head direction" (HD) cells discharge as a function of the animal's head direction in the horizontal plane, independent of the animal's place or behavior (Ranck, 1984; Taube et al, 1990a; Taube, 1998). We quantified the 2D response properties of HD cells using a video-based head tracking system to monitor the rat's directional heading. The direction of maximum response ("preferred direction") lies in a fixed direction and varies from cell to cell. The range of firing is typically about 90°, and decreases away from the preferred direction, as shown in Fig. 1. Responses are independent of 1) pitch or roll of the head up to 90°, 2) head location ("place"), 3) direction of movement, or 4) trunk position relative to the head. Response characteristics are stable over days (cf. Knerim et al, 1995). HD cell firing is largely independent of the animal's movement as long as the head is pointing in the proper direction, and there is little adaptation over time.

To develop effective countermeasures for VRIs and other forms of disorientation experienced in 0-g, it will be important to understand the neural mechanisms underlying spatial cognition. HD cells, by their nature, are important contributors to ones perception of directional heading. Understanding how HD cells respond in different orientation planes, in both 1-g and 0-g, as well as how the vestibular system interacts with HD cells, will provide critical information on how perceived orientation is determined by the brain. Ultimately, this knowledge will enable us to design effective long-term countermeasures for problems of disorientation.

HD cells were initially identified in the dorsal presubiculum (postsubiculum, PoS) (Ranck, 1984:Taube et al, 1990a), and have since been reported elsewhere, e.g. the anterodorsal thalamic nucleus (ADN) (Taube, 1995) lateral dorsal thalamic nucleus (Mizumori & Williams, 1993), lateral mammillary nuclei (Stackman & Taube, 1998), retrosplenial cortex (Chen, et al, 1994), and anterodorsal striatum (Wiener, 1993). Many of these areas are anatomically interconnected. Lesion studies suggest that the lateral mammillary nucleus is critical for directional sensitivity in the PoS and ADN (Taube, 1998).

We have clearly demonstrated multisensory convergence in rodent HD cell responses in experiments manipulating visual and idiothetic cues. For example, the preferred direction of all HD cells is maintained when the animal moves about, even in darkness, but can be simultaneously rotated about an earth vertical axis by appropriate manipulation of surrounding visual landmarks (Taube et al, 1990b), such as a white cue card. Landmark removal does not lead to a cessation of firing, but only to an equal angular shift in the preferred direction of all cells by a random amount. This finding indicates that afferent input driving one HD cell similarly influences other HD cells, and that HD cells within a particular brain area behave as a network and their preferred directions always remain a fixed angle apart (in register) from one other. If the animal is restrained and passively rotated, HD cells often fail to respond, even when the animal's head is in the preferred direction (Taube, 1995). HD cells in the ADN (but not PoS) actually anticipate the animal's future heading by about 25 msec (Blair & Sharp, 1995; Taube & Muller, 1998). These findings show that HD cells also receive motor outflow information. It is clear that HD cells receive vestibular input, as neurotoxic lesions of the vestibular apparatus abolish the directional firing properties of ADN HD cells (Stackman & Taube, 1997). When animals locomote into a novel environment (Taube and Burton, 1995;Taube, et al, 1996)HD cells continue to discharge in a similar direction as they did in a familiar environment. In situations where the spatial information from visual and idiothetic cues conflict in the gravitationally horizontal plane, HD cells usually rely on the visual spatial information.

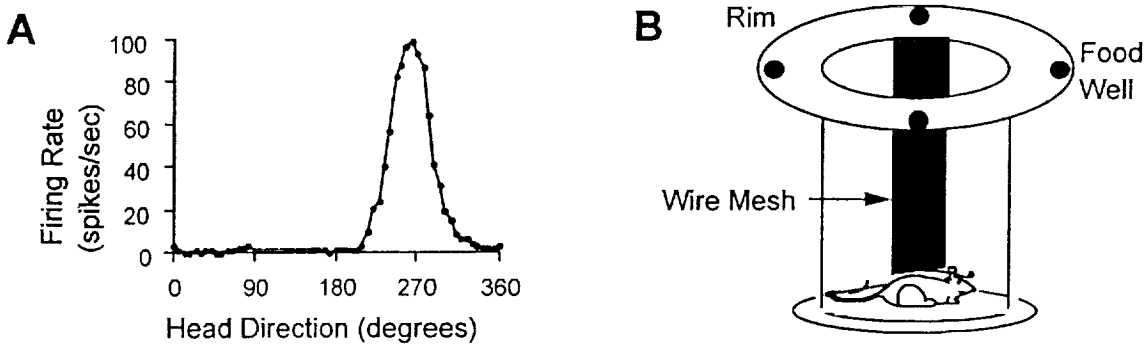


Figure 1. A) Firing rate vs. head direction plotted for a typical HD cell. B) Apparatus used in preliminary wall-climbing experiments.

Shortly before this project began, we conducted experiments to determine how HD cells respond as a rat locomotes into a vertical plane - one which is 90° orthogonal to the floor of our recording cylinder (Stackman, et al, 2000). [Although these experiments were not supported by the present grant, they were inspired by discussions between Drs. Oman and Taube that also later led to the present project.] We also explored whether HD cell activity was affected when the rat was in a second horizontal plane that was significantly separated from, but still in sight of, the first horizontal plane. HD cell activity was recorded in a tall cylinder that contained a wide rim (annulus) around the top with 4 equally spaced food wells (Fig. 1B). A vertical wire mesh "ladder" placed onto the inside cylinder wall allowed the rat to access the

annulus. HD cells were monitored as rats climbed up and down the wire mesh to retrieve food pellets on the floor and annulus. The wire mesh was positioned at 0, 90, 180 and 270° relative to the cell's preferred direction. HD cell discharge properties were similar when the rat locomoted in either horizontal plane (floor or annulus). When the wire mesh position corresponded with the cell's preferred direction (0° position), HD cells continued to fire at peak rates as the rat climbed up the wire mesh, but not when climbing down. With the mesh at the 180° position, cell firing continued when the rat ran down the mesh, but not when it ran up. There was no consistent response when the rat ran up or down a ladder in the 90 and 270° positions.

1.2 Summary of Year 1-3 Research Results

1.2.1 Human Visual Orientation

(I. Howard, G. Hu, H. Jenkin, J. Zacher, R. Allison/ HPL-Crestech –Toronto)

We completed several new experiments on visual reorientation and tilt illusions in 1-G. The rationale and results are summarized below. Data and details on methods are available in the appendixed publications.

1.2.1.1 Relative orientations of body axes, axis of visual polarity, and gravity. We completed a investigation of VRIs as a function of the relative orientation of the body, the visual scene, and gravity by testing subjects in an 8 foot cubic furnished tumbling room (Fig. 2). Results (Howard and Hu, 2000) have been submitted to Perception.

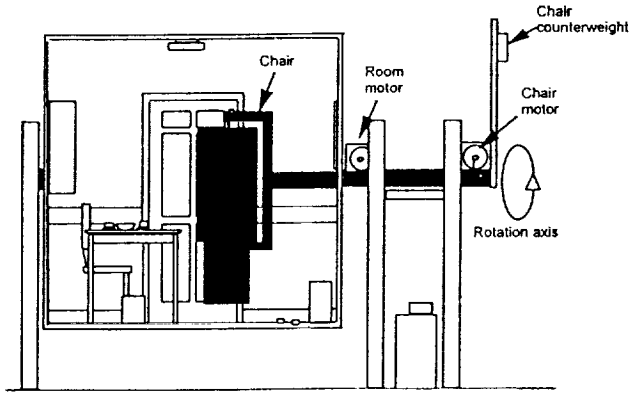


Fig. 2. Schematic of York 8 Foot Tumbling Room

The room is mounted on a horizontal axle. The subject was strapped into a chair and/or bed (www.hpl.crestech.ca/facil.html), which were mounted to a concentric axle so the room and bed could be independently repositioned. Subjects reported the orientation of themselves, the room, and set tactile and visual rods to apparent vertical, for each of 25 relative orientations of the body axis and the furnished room to gravity. In most conditions we used 19 subjects who were susceptible to reorientation illusions. We scored a response as aligned with a given frame of reference only if subjects gave consistent judgments of the direction of 'down' that were within 15° of that frame of reference. A response was scored as a static VRI if a subject judged 'down' to be 90° or 180° to gravity. In most conditions, one or two subjects judged 'down' to be more than 15° from any of the three frames of reference. We classified these as intermediate responses.

We investigated the conditions in which the perception of self orientation to gravity is dominated by gravity, by the visual frame of reference, and by the idiotropic frame of reference (feet perceived as down). Reorientation illusions occurred in all conditions except those in which the room was upright. We found that when subjects were gravitationally supine with the furnished room inclined 90° (so that the polar axis of the room was aligned with the body axis) the majority felt that they were erect in an upright room. The

strong visual polarity cues available in the room overcame conflicting gravireceptor (e.g. otolith) cues. The effect was not due to visual motion or frame effects because the room was motionless, and the frame of the room was vertical. As far as we know it is the first reported case of a static 90 degree reorientation of the subjective vertical in a 1-G situation. Subjects noted that if they extended their arms or legs above their supine body, their limbs felt weightless. The tendency of the limbs to fall back toward the body was ignored. The subjective vertical was along the body axis, so subjects expected their arms to fall towards the feet. Relative to this expectation, their arms felt weightless and seem to float. Objects suspended by thread or hidden supports appeared to float in front of the body. We refer this as a "Levitation Illusion". We noted that a supine subject who saw the vertical room rotate into the 90° orientation were more likely to have a levitation illusion than subjects who suddenly saw the room in the 90° orientation.

VRIs were less frequent when the body was upright than when it was recumbent, supine or prone. The idiotropic frame was used in only 3 out of 142 trials when it was pitted against gravity or the visual frame. The visual frame was used by a majority of subjects when the tilted or inverted room was congruent with the body axis but by fewer subjects when the room and body axis were not aligned. The tendency to judge 'down' as in the direction of the feet (the idiotropic direction) does not override the effects of gravity or the visual frame of reference, but it augments the influence of the visual frame of reference. The prone posture produced the smallest incidence of visually evoked reorientation illusions. This may because the posture of maximum otolith-organ sensitivity is 25° pitch forward or because tactile-proprioceptive cues are more evident for subjects hanging in the prone posture in the apparatus.

These effects are potentially important for astronaut training, clinical, and entertainment industry applications. We will conduct experiments to define what individual characteristics and stimulus related factors potentiate the 90 degree VRI and levitation illusions:

1.2.1.2 Ninety degree static VRI incidence as a function of age In earlier research we had noted that young people are just as likely as older people to experience an illusion of full self rotation when observing a room rotating continuously round the roll axis. In our experiments on static VRIs, however, we noticed that younger subjects seemed less susceptible. We therefore tested 96 subjects (24 in each of four age groups) from 10 to 70 years of age (Howard, Jenkin and Hu, 2000). About 80% of subjects of all ages experienced full illusory self-rotation when they sat erect in the room rotating about the roll axis. When we rotated the subject and room together to an angle of 90°, the percentage of subjects experiencing a static reorientation (levitation) illusion increased from 20% for the 10-year-olds to 80% for the 70-year olds. The percentage of young children experiencing an illusion increased when they were initially supine and the room was slowly rotated into the 90° position. In the last condition, the dynamic illusion of self rotation induced by the initial rotation of the room primed the subjects into believing that they were upright when the room came to rest at the 90° angle. We suggest two possible explanations for age dependency: First, the static cues of visual polarity must be largely learned. Second, vestibular function is known to degrade with age. Our results suggest that older subjects are more sensitive to static displacement of the axes of intrinsic and extrinsic polarity of the visual surroundings. Older people on stairs with no polarized objects (e.g. pictures) in view should be more prone to fall.

1.2.1.3 Manipulations of visual polarity. We built a novel "mirror bed" device (Fig. 3) for inducing static VRIs that allows us to change the visual stimulus more rapidly than in the furnished room: The supine subject viewed a large display through a mirror inclined at 45°. All subjects tested experienced the reflection of a natural outdoor or indoor scene as vertical. We call this a wall response. The head felt upright and, for most subjects, the torso felt inclined between the supine and erect postures. All subjects experienced a blank white surface as a horizontal ceiling with the head and body as supine. We call this a ceiling response. Intermediate responses were rare, although a few subjects gave contradictory responses that were difficult to interpret.

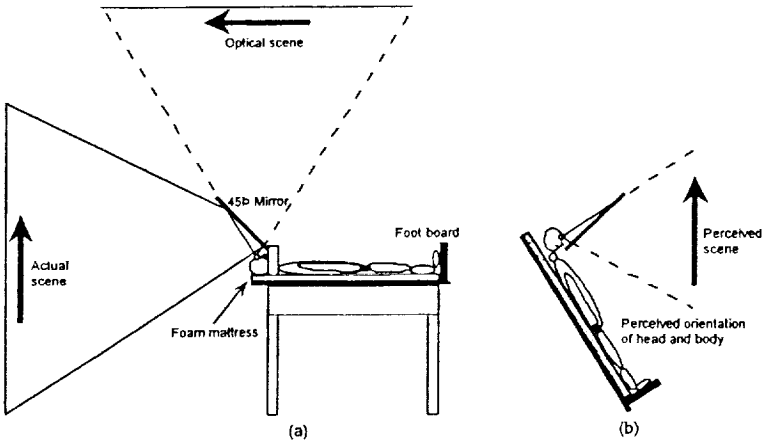


Fig. 3 York 45% Mirror Bed

The mirror bed test paradigm has allowed us to empirically quantify the potency of different types intrinsic and extrinsic visual polarity cues across a group of subjects. Objects with intrinsic polarity (recognizable top and bottom) were presented without visible support or supported on the floor or a shelf. Objects such as balls and boxes were suspended with no visible support or by a visible thread, or placed on one another or on a shelf to create extrinsic polarity. A blank surface induced the ceiling response in all subjects. The percentage of subjects giving a wall response increased as the strength of intrinsic or extrinsic polarity information was increased. Results have been presented (Hu, Howard, and Palmisano, 1999) and a manuscript is in preparation. Our mirror bed paradigm has also been employed in a collaborative vestibular-autonomic investigation conducted jointly by members of the NSBRI cardiovascular and neurovestibular teams: Wood et al (2000) reported transient cardiovascular responses associated mirror bed induced changes in the direction of the subjective vertical.

1.2.1.4 The role of foreground-background relationships. Illusory self motion (vection) is induced by a scene moving beyond a stationary foreground but not by a scene moving in the foreground (Howard and Heckmann, 1989). Using the mirror bed apparatus, we tested the hypothesis that a reorientation illusion is more likely to occur when a polarized visual scene is perceived as a view out of a window than when it is perceived as a picture hanging on a wall. We used a 4-foot by 6-foot photograph of an indoor scene. It was alternatively shown hanging in front of a blank wall in a picture frame or seen through a framed hole in a wall so that it appeared as through a window. In both cases, supine subjects viewed the scene through the mirror at 45°. Significantly more subjects gave a wall response (had a reorientation illusion) when the scene appeared through a window than when it appeared as a picture on a wall.

1.2.1.5 Effects of foot pressure. This year, we mounted a 6-foot long flat bed on the wall of the rotating furnished room. The bed has straps, padding, a spring harness, and a foot plate. When the room rotates 90° the board and subject are brought into a supine posture with the subject looking at the same wall that he faced when erect. The spring harness imposes a downward pressure on the feet. Our goal is to assess the effect of spring harness loading on levitation illusion incidence. Similar harnesses have been used in 0-G research (e.g. Young et al, 1993; Oman et al, 2000), but their effects on the subjective vertical in 1-G are not known.

1.2.1.6 Field of View, Head Motion, and Scene Speed on Vection and Tilt. With NSBRI support, we completed an experiment (Allison et al, 1999) in the tumbling room to investigate the effects of field of view, head motion, and velocity of room rotation on the magnitudes of roll vection and illusory self tilt. We wanted to understand how field of view and head movement restriction, typically the result of using head mounted displays, influenced VRIs. The view of the room was masked down by an aperture of various diameters and the head was either restrained or free to move. The furnished room rotated at various velocities about the roll axis of the stationary erect subject. Subjects rated illusory self motion on a 5-point scale and reported whether they experienced alternating tilt of the self or complete illusory rotation. The results showed that the incidence of complete self rotation in the rotating furnished room increased as field

size increased from 20° to full-field viewing and as the velocity of room rotation increased from 15 to 30°/s. Freedom of head movement had no reliable effect.

1.2.2 3D Spatial Memory and Learning

(C.Oman, J. Richards, A. Beall, A. Natapoff/MIT; W. Shebilske/Wright State University; Travis Tubre, Tim Willis, and Amber Hanson /Texas A&M University)

1.2.2.1 Effects of Gravitational Position and Display Type. Our goal has been to better understand the processes of 3D spatial memory, and to apply that knowledge to develop effective countermeasures for the problems summarized earlier. The objective of our first series of experiments was to understand how quickly and how well subjects learned to perform a generic spatial memory task analogous to that confronting a crewmember in a space station node. Since we were interested in developing preflight training countermeasures for astronauts, we wanted to know if performance in real and virtual environments was equivalent, and whether the subject's body orientation with respect to gravity was important. Results (Oman, et al 1999 ; Shebilske et al, 2000) have been presented and a manuscript (Oman, et al, 2000) has been submitted to Spatial Cognition and Computation. Briefly, a group of subjects (n=73) were tested at TAMU in a cubic chamber (Fig.4), and a second group (n=24) were tested at MIT in an equivalent virtual environment, wearing a color stereo HMD (Fig. 5).



Fig. 4 Physical display system, erect condition

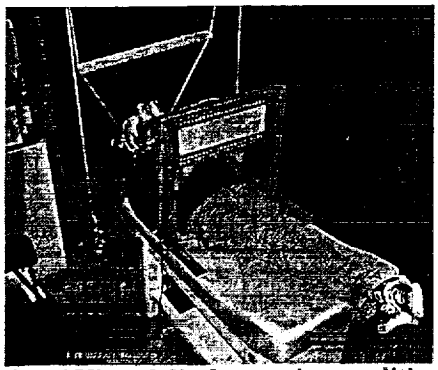


Fig. 5 Virtual display, supine condition, showing head support system, and response keyboard.

The basic task was a trial-by-trial learning paradigm, comparable to the card game "Concentration," except played in 3 dimensions: A picture of an object was presented at the center of each wall. Subjects had to

memorize the spatial relationships among objects on the six walls of the node, and predict the relative direction of a target object if their body was in a different roll orientation, and/or they were facing a different wall. Their performance was measured in terms of response time and percent correct indication. After they responded, subjects were shown a view of all six objects on the interior walls from this relative orientation and allowed to confirm their answer and briefly study the object configuration before continuing with the next trial. The timeline for each trial is shown schematically in Fig. 6.

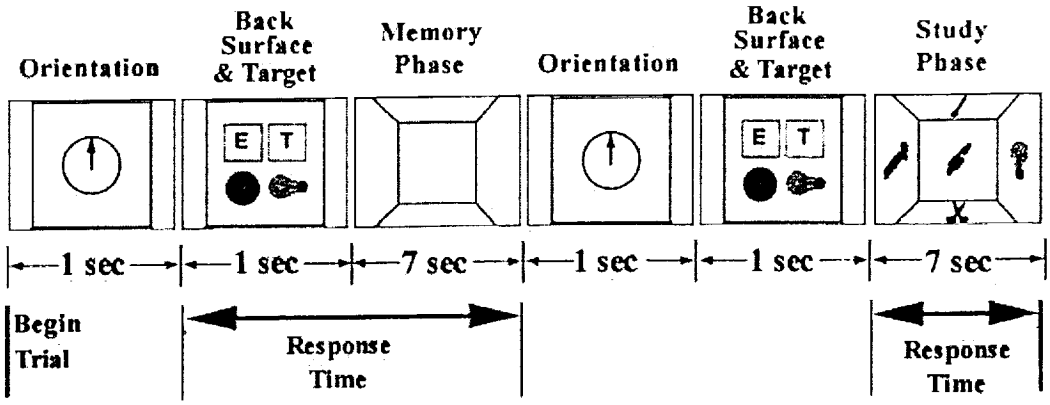


Fig 6. Timeline for each trial. Subject is shown imaginary orientation clockhand, back surface (E) and target (T) objects, then during "memory" phase indicates the relative target direction to the imaginary target while in darkness. Orientation, back surface and target commands are repeated, and then during "study" phase, all 6 targets are shown. Subject must find target, indicate relative direction, and then study array for remainder of time available.

Half the subjects in each group began training erect, and then switched to the supine position after 24 trials, while the remainder did the reverse. Most subjects were able to achieve high accuracy within 20 trials from a given viewpoint, regardless of roll orientation, and could quickly learn a second viewpoint with equal or greater ease. Quantitatively similar results were obtained for the real and virtual environment subject groups. Although our tilted room experiments at York (Sect. 1.2.1.1; Howard and Hu, 1999) indicate that VRIs are facilitated when subjects are in a horizontal position, in the present experiments, physical body position had only a minor effect on performance. Exit questionnaire responses and interviews suggested most subjects used a combination of declarative mnemonic rules and mental visualization techniques, e.g. a "spatial framework" (Bryant and Tversky, 1999). The majority imagined the node as stationary, and rotated themselves within it. Subjects said task was something "done in your head", which may be why gravitational body position and display details were not particularly important. However, task performance of both subject groups was significantly correlated with scores on conventional paper-and-pencil tests of field independence (GEFT, Witkin et al, 1971) and 2 and 3D figure rotation ability (Eckstrom et al 1976). We concluded that despite limitations in field of view, resolution, tracking delays and other factors, head mounted virtual reality displays can be employed for this type of spatial memory training, and that subjects may remain in the physically more comfortable and practical gravitationally erect position.

1.2.2.2 Arbitrary Viewpoints, Multiple Environments, Strategy Training, Skill Retention, Body Axis effects.

A second series experiments in a virtual node were completed at MIT during the past year (Richards, 2000; Richards and Oman, in preparation). The training paradigm was changed in several significant ways: First, rather than use a "clock hand" to specify the subjects orientation in each trial as in our first experiments, the objects opposite and below the subject were shown, followed by the identify of target object but not its direction (Fig.7, 2nd panel from left). The subjects then inferred their orientation and indicated the relative direction to the unseen target object. Second, we wanted to know if subjects could visualize the environment facing any of the six node surfaces, not just two as in our initial experiments. Hence, after four initial trials in a fixed "baseline" orientation, and eight more trials facing a second surface in various roll orientations, the subject performed 24 trials facing all six surfaces in a variety of different orientations.

Presentation order was pseudorandomized but balanced by surface and relative target direction. Third, we wanted to know if learning one environment enhanced a subject's ability to learn another environment, so subjects were then immediately retested using a different set of objects. Do subjects "learn-how-to-learn"? (Each "object" consisted of four identical pictures of a familiar animal, each rotated by multiples of 90 degrees and symmetrically clustered so the aggregate could be easily recognized from any orientation, and provided no pointer cues to the other five objects. The order of presentation of the environments was reversed for half the subjects, to control for intrinsic differences in "learnability" of object sets.). Fourth, we wanted to know whether strategy training is helpful. In our earlier experiments, we wanted to learn what strategies subjects used, so they received no formal training. In this series, we instructed half the subjects to memorize opposite pairs of objects, and to remember object triads (corners), while the remaining subjects received placebo instruction as a control group. (Different written instructions were presented to each group via computer. The subject groups were matched using two and three dimensional figure rotation test scores.) Finally, since we were developing the paradigm as a generic 3D spatial memory training procedure for astronauts, we wanted to know how long after training the knowledge and skills were retained. Hence, our subjects returned one, seven and thirty days later. Retention of their configurational knowledge of both previously learned environments was tested by asking them to pick-and-place objects from a palette onto the appropriate surfaces of the node. Their spatial memory ability was then retested in the second environment facing all six surfaces in arbitrary directions (24 trials), but subjects were not shown the object configuration between trials. Response time (RT) and percent correct learning curves were measured on all four days, while configurational knowledge was tested on the last three.

Twenty four subjects were tested. Both the strategy training and control groups learned to do the task within 36 trials in either test environment, ultimately when facing any of the six surfaces in an arbitrary orientation. Both groups responded faster in the second environment than the first (particularly the strategy group). The strategy group showed superior percent correct and RT scores for above/behind targets, and generally better configurational knowledge. The response time result is consistent with Tversky's (Franklin and Tversky, 1990; Bryant et al, 1992) findings using environments presented using written narratives. The 8 subjects who reported using mental imagery had higher scores on figure rotation tests and significantly higher percent correct for left/right targets. Retention of configurational knowledge and spatial ability for both groups was good when tested at 30 days (21 days since last test). As in our first study (Sect. 1.2.2.1), we found that performance of the control group was significantly correlated with paper-and-pencil test measures of field independence and 2/3D figure rotation ability. Strategy training appeared to help those who had measurably poorer mental rotation skills, and those who denied using mental imagery. Exit questionnaires suggested that most of the control group eventually discovered and used the "pairs" strategy which had been taught to the training group. The training group disliked the "triad" strategy, which required application of a right-hand-rule.

That ability transferred to a new environment, and was retained over several weeks is important if three dimensional spatial memory training is to be used as a countermeasure for astronauts. Our subjects clearly differed in their ability to do 2D and 3D mental rotation, and in their ability to visualize the environment. In this relatively short training session, it is unlikely we taught anyone to use mental imagery. Rather, we simply encouraged those who had the ability to use it. Subjects learned to employ a combination of taught and self-developed declarative rules, supplemented by mental imagery, particularly when dealing with targets on their left and right. Ability to employ visualization in this situation ought to depend on two and three dimensional mental rotation ability, and such a correlation was in fact observed. Paper-and-pencil tests of mental rotation ability may allow us to tailor the visualization/strategy emphasis in training to individual subjects. The "baseline orientation" and "memorize opposite pairs" strategies were successful – whether taught or self-discovered. Theory suggests that learning corners should be useful, but our method of remembering triads using a right hand rule was cumbersome and needs improvement.

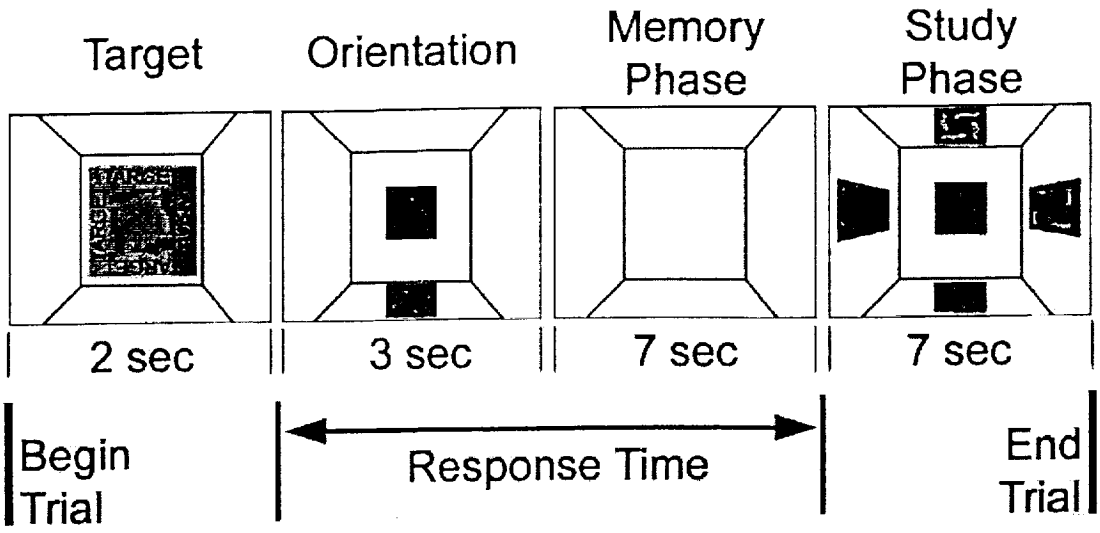


Fig 7. Schematic of Expt. 1.2.2.2 Experiment timeline for each trial. Subject is shown target identity, and the direction of surfaces in front and below. Then, during the "Memory" phase the subject has 7 seconds to indicate relative target direction in body axes. Next, during "Study" phase, all 6 targets are shown. Subject finds target and then studies array for remainder of time available. Compare with Fig. 6.

1.2.2.3 Blocked vs. Random Training, Model vs. Full Scale Environments.
Two other experiments have been underway at TAMU/Wright State using a physical node mock up. The first experiment specifically contrasts the benefit of random sequencing of training trials over blocked sequencing. The random sequence requires trainees to practice mental manipulations on every trial. The other sequence does not because it is blocked with respect to the simulated perspective. Eighty subjects have been tested. On theoretical grounds (Bjork, 1994), we expected random sequencing would ultimately enhance performance better than blocked presentations despite the short-term challenge. Our results supported this prediction. Subjects in the blocked condition apparently did better initially because they could answer without doing mental rotations. Subjects in the random condition did better in learning a second module, probably because they improved a generalizable spatial memory skill. A manuscript is in preparation.

What is the nature of the spatial memory skill that improves in the physical node mock up? How does this skill relate to the spatial memory skills that astronauts need to naviagate in the international space station? How do these skills relate to the spatial memory skills that we use on earth? The second experiment addresses these questions. It compares performance using a small scale physical model of a node. In one condition, the model remains in one position, and the subject physically moves around the model and looks in through a side with their head in the appropriate orientation before responding, Instructions are as in our first experiments (Shebilske, et al, 2000; Oman et al, 2000) The second condition is similar except the subject remains in one place, and experimenter rotates the cube model behind a curtain out of view of the subject. This requires the subject to visualize the cube in a new orientation in a manner analogous to the immersive experiment. By moving to the correct position in the first condition, subjects indicate that they understood the instructions. The two conditions are equivalent in all other ways including the stimulus that the subject sees in the box, the sequencing of stimuli, the temporal spacing of stimuli, and the required pointing response. Both conditions require equivalent mental transformations of the relationship between the subject and the box. We hypothesized, however, that highly overlearned, automatic processes perform the transformation when the subject moves and the box remains fixed and that conscious mental rotation processes perform the transformations when the subject remains in one position. The transformation processes used in the first condition are part of spatial memory skills that people utilize in everyday situations to automatically keep track of their position relative to objects in their environment. We believe that the transformation processes used in the second condition are part of spatial memory skills that people use when they consciously manipulate mental representations of spatial relationships as when they read or listen to descriptions of spatial relationships or when they become disoriented in unfamiliar environments

such as scuba diving. The presence or absence of a secondary task was factorially combined with the other two conditions to investigate the automatic versus conscious nature of these processes. The secondary task was counting backwards by three between the time of seeing the stimulus and making the response. Both groups were also given the same paper and pencil tests of mental rotation ability that we have used in our previous experiments. Preliminary data from 20 subjects indicates that the simulated rotations result in gradual learning comparable to the immersive blocked condition, whereas learning is very rapid (2 or 3 exposures) in the physical movement condition. The secondary task disrupts the simulated condition, but has little or no effect on the physical movement condition. Based on our previous results, we expect performace in the simulated rotation condition to correlate with performance on the paper and pencil tests. We do not expect this correlation in the physical movement condition. All conditions require analogous mental transformations to relate one trial to another. Therefore, the study provides controlled comparisons of variables that affect those processes. Clearly, the
processes in the physical condition, which are similar to those used on earth, are very different from those in the simulated conditions, which are analogous to those that astronauts have to use if they find themselves disoriented. Understanding these will guide the development of training countermeasures. This experiment will be completed and submitted for publication early next year..

1.2.3 Neural Coding of Spatial Orientation

(J. Taube, R. Stackman, M. Tullman, J. Calton/Dartmouth; C. Oman/MIT)

1.2..3.1 HD cell responses in 3-D under 1-G and 0-G conditions. As described above, astronauts working in 0-g often experience visual reorientation illusions (VRIs) that sometimes trigger bouts of space sickness and often lead to reaching errors. Our goal was to better understand orientation and navigation in 1-g and 0-g by determining how visual, gravitational, and other cues anchor the 3-dimensional response characteristics of HD cells. Except for our wall-climbing experiments described below, all previous HD cell research has involved locomotion in an Earth horizontal plane. What does the 3D response characteristic of an HD cell look like? Do the 2D response curves remain as invariant in 1-g when an animal crawls on a wall or the ceiling, as one expects? Or does the response change planes, so it aligns with the plane of locomotion? Do HD cells continue to respond in 0-g parabolic flight? Does the 3D response characteristic become more labile in 0-g, since the gravitational "down" reference is absent? Does the azimuthal response plane align with the plane of locomotion or with the visual reference frame?

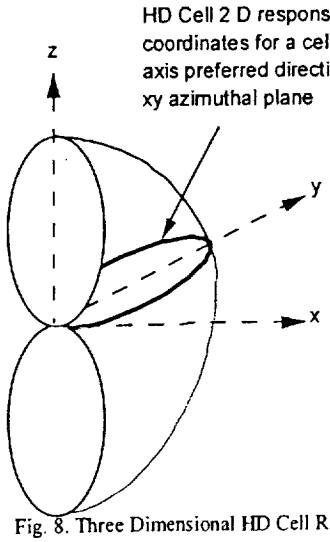


Fig. 8. Three Dimensional HD Cell Response Model

Based on the 2D response characteristics of HD cells, and our wall climbing experiments (Stackman, et al, 2000), we hypothesized that the 3D response characteristics of an HD cell can be described in spherical coordinates by the hemi-toroidal surface shown in Fig. 8. In this figure, the positive y axis represents the preferred direction of a 2D HD cell response (in polar coordinates). Under normal conditions in 1-g, the z axis is aligned with the gravitational vertical, and the x-y plane is horizontal. The length of the vector from the origin to the surface defines the magnitude of the HD cell response as a function of the animal's 3D directional heading represented by the vector's direction. For example, consider an HD cell which responds maximally when the animal's head faces in the positive y axis direction. HD cell responses are known to be independent of head pitch and roll up to 90°. The model predicts that the cell will discharge at its peak rate when the rat's head is oriented anywhere along the y-z plane, as long as the animal's head orientation contains a positive y-axis

component. Thus, the cell will continue to fire if the animal climbs the North wall (defined as the wall in the x-z plane by the positive y axis), but not the South wall. If the animal climbs the West wall, the cell will respond whenever the animal's right side is down, but not the left, and conversely on the East wall. This model can be used to predict the 2D response for any plane in the experiments described below.

1.2..3.2 HD cells responses when the rat is upside down on the ceiling. Food restricted rats were trained to run around a 4'x4x'12" wide ferris wheel-like square track that was oriented vertically (see Figure 9). Each surface contained a wire mesh (10" wide) to allow the rats to grasp and climb on. The floor surface was divided into two compartments. When in one floor compartment, the only way the rat could reach the other floor compartment was to climb up the wall, traverse the ceiling, and then climb down the other wall. Food was available in only one floor compartment and the rat started in the other floor compartment. The amount of food reward was limited so that once the rat reached the goal, it had to run back to the original compartment to get additional food. Thus, the rat learned to shuttle back and forth between the two floor compartments by traversing the walls and ceiling. The apparatus was centered in a white colored square room that contained a large black curtain hanging from one wall. The purpose of the curtain was to provide a salient orienting cue for the rat. The entire apparatus could be rotated in the azimuthal plane so that we could examine HD cell responses either when the preferred direction was aligned with the plane of the apparatus or when the preferred direction was orthogonal to it. In addition to the ceiling mounted video camera, we had three additional cameras mounted to view the ceiling and the two vertical ladders. The task was not an easy one to train rats to perform, especially climbing upside-down on the ceiling. After several variations in shaping paradigms we have settled on one which enables us to train a rat in 2-3 months. We have currently recorded from about 10 HD cells in 3 rats. Results show two categories of responses. Some HD cells show robust direction-specific firing while the animal locomotes upright on the floor and walls, but lose their direction-specific firing when the animal is locomoting upside-down on the ceiling. For a second class of cells, the directional tuning is maintained in a world-centered reference frame when the animal is locomoting upside-down. Thus, these cells continued to discharge in the same preferred direction with respect to the room as they discharged when the rat was on the floor. Thus far only one response type has been observed in an individual animal, suggesting that some rats may be better at orienting themselves on the ceiling than other rats. As with the first category of HD cell responses, firing on the walls was dependent on the direction from which the rat approached the wall. If it approached it from the cell's preferred direction, then cell firing continued as the rat locomoted either up or down a particular wall. Conversely, if the rat approached the vertical surface from a head orientation that was not facing the cell's preferred direction, then the cell didn't fire as the rat traversed the wall up or down. These results could be significant given the disruption of HD tuning observed in animals during upside-down locomotion during 0-G parabolic flight:

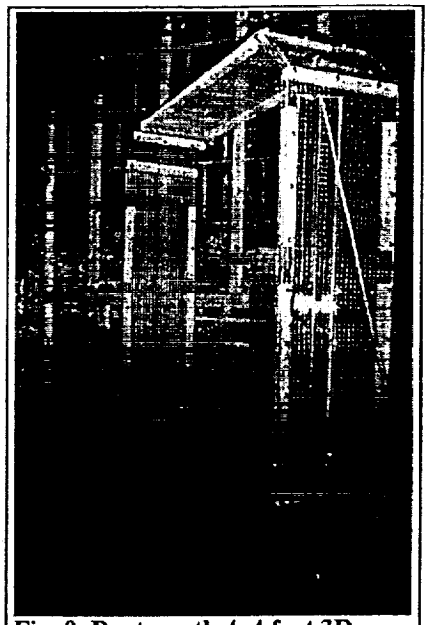


Fig. 9 Dartmouth 4x4 foot 3D wire mesh track, with rat in left side compartment

1.2.3.3 HD cell responses in 0-g Parabolic Flight – an animal model for human 0-G visual reorientation. To understand some of the neural mechanisms underlying disorientation in space flight, over the past 18 months we have performed two series of experiments recording from HD cells under 0-g conditions in parabolic flight. The experiments were designed to characterize and compare HD cell responses in 3-D under 0-g, 1.8g, and 1g conditions. In particular, we were interested in determining whether HD cells would continue to respond in 0-G, and whether they occasionally would shift their preferred direction 180° when the rat was upside down on the ceiling. This type of response would be consistent with the rat undergoing a VRI, as when astronauts report that they sometimes perceive themselves as upside-down and their directional heading 180° reversed.

A clear Plexiglass rectangular cage (Fig. 10) was constructed that had wire mesh covering the floor, ceiling, and one wall, and mounted on a pedestal inside NASA's KC-135 parabolic flight aircraft. The interior of the cage was visually up-down symmetrical and curtains were used to limit surrounding environmental cues from the cabin were arranged so that up-down visual cues were relatively ambiguous. Because the 0-g conditions obtained in parabolic flight are brief (20-25 sec), when the rat was not locomoting itself, we gently nudged the rat into a variety of different orientations during each parabola. The position and orientation of the animal was recorded by three video camcorders, and HD cell activity was concurrently recorded on the camcorder audio track. Each HD cell was monitored across about approximately forty 0-g parabolas.

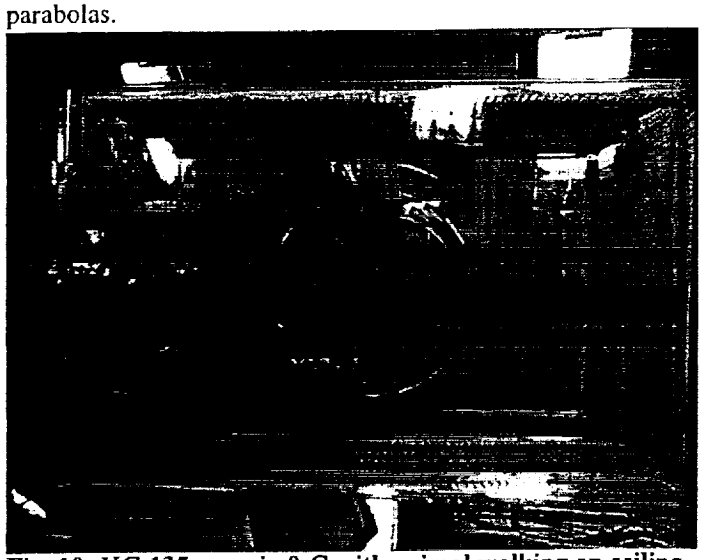


Fig. 10: KC-135 cage, in 0-G with animal walking on ceiling

Following completion of the flight, HD cell activity was correlated with the rat's head direction during the different phases of parabolic flight. The animal's instantaneous head direction in the plane of locomotion was classified into one of eight 45° bins by a human observer. Audio track HD cell activity was quanitified using a spike counter. Variables examined over the various phases of parabolic flight included: 1) the directional specificity (preferred-direction) of the recorded cell, 2) the overall shape of the firing rate/head direction tuning curve, i.e., does the tuning curve follow the typical triangular model, or does it follow a different (eg., rectangular) model. 3) changes in background firing rates and peak firing rates. In addition to examining these issues over the various phases of parabolic flight, These variables were assessed during the 1.0g, 1.8g (pullout) and 0.g phases of flight as the rat locomoted the different surfaces of the apparatus (floor, wall and ceiling), and while the experimenters themselves were oriented upright and upside down.

We have now recorded from 7 HD cells (from ADN thalamus) across 6 rats with generally consistent responses observed across all cells. A preliminary report (Taube, et al, 1999) has been made and a manuscript is in preparation. All cells maintained their direction-specific discharge when the rat was on the cage floor during the 0-g and 1.8 g pull-out periods. The cells' preferred directions were also maintained when the rat crawled on the wall in 0-g. However, direction-specific firing was usually disrupted when the rats were placed on the ceiling and there was no single direction at which the cells fired. There also appeared to be an increase in background firing. The loss of directional tuning upside-down on the ceiling occurred whether the investigators were upright or inverted with respect to the aircraft. At least two cells consistently responded during some parabolas when the rat's head was oriented 180° opposite the preferred direction of the cell when the rat was on the floor. These responses suggest that during these particular parabolas the rats maintained a normal allocentric frame of reference in 0-g and 1-g when on the floor or wall, but when placed on the ceiling in 0-g, the rats appeared to be disoriented (as judged by the loss of directional specificity in HD cell firing). The occasional reversal of HD cell preferred direction across the cage axis of symmetry indicates that the rats may have experienced sensations comparable to what humans would describe as a visual reorientation illusion during these parabolas. When humans roll inverted in 0-G parabolic flight, they say the ceiling of the airplane seems like a floor, and the left-right axis seems

strangely reversed. We believe this is the first demonstration in an animal model of a limbic correlate of a human 0-G spatial orientation illusion.

It is also interesting to note the similarities and differences in the results of the experiments described in 1.2.3.2 vs. 1.2.3.3. When the rats are upside-down on the "ceiling" - in both cases the most frequent results was the loss of direction-specific firing, suggesting a general loss of directional sense when upside-down. However, in 0-g parabolic flight, but not in 1-g, reversed responses were sometimes observed on the ceiling, consistent with the hypothesis that the plane of HD cell sensitivity had reoriented by 180° onto the new surface of locomotion, whereas this did not occur in 1-g.

2. Implications of Project Findings for Future Research

2.1 Human Visual Orientation

Our research on human visual orientation have shown that adults become more susceptible to the effects of static visual cues with age, and defined a practical scientific technique for quantitfying the strength of "gravitational polarity" and "frame" of visual objects. Our results provide an objective basis for updating NASA human factors standards (e.g. 3000/8.4.3) relating to space station architecture, workstation layout, and visual verticals. Our findings also suggest that pictures of polarized objects on the interior walls of spacecraft might prevent disorienting VRIs in astronauts. In the public health domain, our results indicate that older people remain sensitive to optic flow but become particularly sensitive to static displacement of the axes of intrinsic and extrinsic polarity of their visual surroundings. Older people on stairs with no polarized objects in view should be prone to fall. Pictures of polarized objects on the walls beside the stairs should help maintain balance.

Our discovery during the previous grant period that compelling static visual scenes can reorient the subjective vertical by 90 degrees or 180 degrees has opened a number of important new lines of research. Our method of using a mirror-bed to produce a 90 degree VRI has already proven useful in research on visual-vestibular-autonomic connections (Wood, et al, 2000). When a supine subject experiences a 90 degree VRI, the absence of a gravity component along the body axis makes limbs feel weightless if extended or supported ("levitation illusion"). This may also provide a useful tool to investigate and partially simulate Otolith Tilt Translation Reinterpretation (Parker, et al, 1984, Young, et al, 1984), as well as the effects of the subjective vertical on motor control. We plan to investigate how the illusory expectation of gravity along the body's z-axis influences the perceived orientation of self, causes oscillopsia and visual lag, and disturbs oculomotor responses to head and body movement. This will ultimately help us understand why head movements in zero G can be disorienting and are nauseogenic. The 90 degree VRI technqiue be developed into a countermeasure for pre-adapting subjects to some aspects of the visual-motor conditions found in zero-G.

2.2 3D Spatial Memory and Learning

Taken together, our spatial memory experiments have demonstrated that learning in using real, virtual, and model environments is similar, that the gravitational orientation of the subject is not of major importance, and that subjects can perform both reverse (imagined body orientation) and forward (inferred body orientation) tasks, analogous to what astronauts face in a space station node. That training improves performance in a second environment containing different landmarks indicates that teaching generic strategies and providing practice is helpful - that subjects "learn how to learn". Our current training procedure requires about 2 hours of training time. Participants come out of the experience feeling that they have learned some useful tricks for orienting in a three-dimensional environment, and this ability is retained for at least 21 days. We are currently working on extending the basic paradigm to measure spatial memory across two previously learned modules, one of which is unseen. We want to see if coalignment of the baseline orientations of the modules is important when making judgements across multiple modules.

Based on reports from MIR crewmembers, we believe that it will be. We have proposed an experiment during the next grant period to find out.

In the course of conducting our research, we have realized that although we have had an opportunity to debrief a number of astronauts in person, and anecdotal reports have appeared in the popular literature (books by Burroughs, Linnenger) there is no quantitative data on the incidence of VRIs and spatial memory problems on Extended Duration Orbiter, NASA-MIR, or early ISS flights. NSBRI is supporting one of our project researchers, Mr. Jason Richards, to work for 3 months this fall at NASA-JSC in the laboratory of Dr. Jon Clark, reviewing medical debriefs and crew transcripts, interviewing other astronauts, and compiling statistical data. Results also support Dr. Clark's Neurological Function Integrated Project Team activity in operational medicine. Dr. Oman and Dr. Tom Marshburn, a NASA-MIR flight surgeon, are consulting on the project from Boston.

Astronauts have the opportunity to develop "baseline" spatial frameworks for individual Shuttle and ISS interiors as they train in Building 9 simulators at NASA JSC. However, their opportunity to develop an allocentric spatial framework which allows them to make accurate spatial judgements about the spatial relationships between modules in emergencies is very limited. One important application of our research paradigm is potentially in the design and evaluation of emergency escape route markings and systems of visual landmarks within modules that help crewmembers identify the principal axes of the ISS. Preliminary discussions with some of our JSC human factors colleagues (Operations Habitability Group/SF) are underway in this regard.

Our results have provided new insights on how spatial memory is constrained when the entire visual environment is rotated about other than the principal body axis. Our findings also potentially apply to understanding disorientation in other situations on earth, as when flying an aircraft, flight simulator, in SCUBA diving, or in certain virtual reality applications.

Our ultimate goal is not merely scientific understanding, but the development of a methodology/pedagogy for training and evaluating 3D spatial orientation and spatial memory skills in astronauts for specific ISS and Shuttle missions. Such a training program necessarily must be managed and conducted at NASA. It might utilize both workstation class computers for generic training and – if mission specific training is important - more elaborate facilities such as JSC's Integrated EVA/RMS VR Simulation Facility ("VR Lab"), where multiple crewmembers currently rehearse EVA using photorealistic spacecraft models rendered via a powerful SGI Reality Monster graphics computer. Ultimately it will be important to determine how effective mission (environment) specific training is relative to generic visual orientation training. The obvious advantage of generic training is that it is quicker and cheaper than mission-specific training. The latter requires more programming time to model actual environments in a realistic way, and crewmembers will need to memorize – and interrelate – sets of visual landmarks in multiple modules.

2.3 Neural Coding of Spatial Orientation

Our experiments (Stackman, et al, 1998; Stackman et al, 2000; Calton et al, 2000) in 1-g have demonstrated how gravity anchors the orientation of the hemi-toroidal HD response characteristic in a rat animal model. Our parabolic flight experiments (Taube, et al, 1999 and in preparation) have shown that even when gravity is absent, the hemi-toroidal response characteristic usually remains anchored by visual cues, since HD cells normally show the stable preferred directions in 0-g as in 1.0 and 1.8-g and 1.8g when locomoting on the floor and wall. That HD cells occasionally show directional reversals when moving about on the ceiling in 0-G of the up-down visually symmetric test chamber provides first demonstration of the probable neural basis of human 0-G visual reorientation illusions. We believe that one function of HD cells is to stabilize (and occasionally reorient) the firing patterns of hippocampal place cells, which have been described as the animal's internal "cognitive map" of the local environment. Corroborating evidence has recently come from related Neurolab space shuttle experiments on 0-G place cell responses conducted by McNaughton, Knerim and colleagues (Knerim et al 2000). They noted that the otherwise paradoxical changes in 0-G place cell response fields seen in certain of their animals while traversing a visually ambigous 3-D "Escher staircase" track could be directly explained if the HD cell responses had reoriented

as we have shown. Their original working hypothesis for the experiment design had not considered the possibility of HD cell coordinate frame reorientation. Taken together, these experiments provide important insights on the fundamental role played by gravireceptors in stabilizing our sense of place and direction not only in astronauts, but also in vestibular and Alzheimer's patients. Funding from NASA has allowed us to pursue the role of gravity as a major theme, and provided access to the unique facilities required for parabolic flight experiments. This part of the project has required only a relatively small fraction of the total resources, but has helped all the members of our project team extend their thinking from the cognitive to the cellular level. Our results have suggested several new research questions. For example, when astronauts experience a VRI with their feet on the "ceiling" of the spacecraft, does it matter whether they rolled or pitched to the inverted position ? What mechanisms cause HD cell directional specificity to be lost ? When HD cells lose directional sensitivity, does the animal show disoriented behavior ? Of what kind ? Does semicircular canal plugging abolish HD cell response in animals ? What happens to HD cell response which nucleus prepositus units are lesioned ? (Nucleus prepositus in the brainstem plays an important role in the integration of angular velocity information to angular position at a much earlier level). Over the next several years, we hope to be able to find answers to some of these questions.

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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

Research Team: *Neurovestibular Adaptation*

Project Name: *Advanced Techniques for Assessment of Postural and Locomotor Ataxia, Spatial Orientation, and Gaze Stability.*

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EXECUTIVE SUMMARY

Adapting to microgravity is not the only balance difficulty astronauts face. Major postflight problems include difficulties with standing, walking, turning corners, climbing stairs and other activities that require stability of upright posture and gaze. These difficulties inhibit astronauts' ability to stand up, bail out, or escape from the vehicle during emergencies and to function effectively when leaving the space/shuttlecraft after flight. Thus it is important to understand the cause of these profound impairments of posture, gaze and locomotion stability in many returning astronauts (and in vestibular patients), and determine how they can be quantified.

Any developed countermeasure must be tested to determine its effect on gait stability, particularly under those conditions that are most troublesome following spaceflight. These countermeasures must be tested with valid and reliable tools.

This project's aims were to develop quantitative, parametric approaches for assessing gaze stability and spatial orientation during normal gait and when gait is perturbed. It has produced two new findings that are key to the understanding of human locomotion and a novel way of characterizing locomotor disturbances that are described below.

c. Understanding movements of the eyes, head, and body during locomotion.

The ability to see objects clearly during locomotion is important for preventing collisions, falls, trips, and clumsy maneuvers. Clear vision requires that the image of the visual object of interest remains steady upon the retina. This ability to stabilize a retinal image is know to be compromised upon a change in g level after a prolonged exposure to a previous g level (for example, Earth return from a micro g orbit). To understand just why there is blurred vision in returning astronauts, it is necessary to understand jointly during locomotion: the motion of the head in space, the motion of the eye in the head and the relationship of the visual target to the person. Two important results from our research involve the relationship of head motion to walking speed and the relationship of eye motion to visual target distance.

Trunk and head movements were characterized over a wide range of walking speeds to determine the relationship between stride length, stepping frequency, vertical head translation, pitch rotation of the head, and pitch trunk rotation as a function of gait velocity. The results suggest that two mechanisms are utilized to maintain a stable head fixation distance over the optimal range of walking velocities. The relative contribution of each mechanism to head movement depends on the frequency of head movement and consequently on walking velocity. From consideration of the frequency characteristics of the compensatory head pitch, we infer that compensatory head pitch movements may be produced predominantly by the angular vestibulo-collic reflex (aVCR) at low walking speeds and by the linear vestibulo-collic reflex (lVCR) at the higher optimal speeds of walking

Eye and head movements were also characterized during locomotion while the subject viewed near and distant targets. For near targets eye velocity was essentially in phase with head pitch velocity . Eye velocity increasingly lagged head pitch as target distance increased, and was compensatory at 2.0 m. For far targets, the gain and phase of eye *re* head pitch velocity indicated that the angular vestibulo-ocular reflex (aVOR) was generating the eye movement response. For near targets, decreasing target distance would augment the lVOR gain, and the eye velocity phase suggested that the linear vestibulo-ocular reflex (lVOR) was generating the vertical eye movement response.

The significance of the studies is that we have gained considerable insight into the compensatory and orienting functions of the vestibulo-collic and vestibulo-ocular reflexes during locomotion. Through this has come the development of countermeasure assessment criteria which can now be applied in studying behaviors that have proven to be difficult following exposure to microgravity.

d. Understanding the orientation mechanism during locomotion.

One of our working hypothesis was that the above-mentioned "profound impairments of posture, gaze and locomotion stability" are caused by alterations in compensatory and orientation mechanisms that are generated in the central vestibular system from motion inputs. During exposure to altered gravity, the motion inputs from the otolith organs are "distorted" compared to the on-earth conditions. These distortions, in turn, cause both inappropriate body head and eye movements and an altered sense of orientation, which degrades stability during locomotion.

We compared motions of the body during walking along a straight line with body motions while walking along a curved path. In the latter condition subjects accelerate in toward the direction of the curve which introduces an inertial component which may or may not effect measures of their body orientation in space. Our data show that compensatory eye, head and body movements stabilize gaze during straight walking, while orienting mechanisms direct the eyes, head and body to tilts of the resultant of gravitational and centripetal acceleration in space during turning. This finding in normal subjects can now be compared to subjects with known impairments in their balance system or to returning astronauts to determine whether or not such individuals can successfully align parts of their bodies in an appropriate way while turning.

e. Characterizing the recovery trajectory to disturbances of locomotion.

Analysis of perturbed gait provides a means of evaluating the success of measures designed to counter loss of balance due to disease or exposure to microgravity. We measured several body segment variables (head, sternum, legs) and especially their trajectories in

response to mechanical perturbations that were precisely delivered in time, magnitude, and direction to the foot.

In normal individuals, the recovery trajectory show a large initial displacement due to the disturbance and subsequently crosses the baseline at the second step to show a slight underdamped response at the third step with a return to, or near, the baseline by the fourth step. In contrast, the recovery trajectory for a pilot vestibulopathic patient shows a distinctive different pattern which takes several more paces to recover.

The development of an experimental paradigm that introduces a calibrated disturbance to the foot during the support phase of normal locomotion provides a means for the objective quantification of locomotor response dynamics that are known to be altered in astronauts upon return from exposure to microgravity but for which no current test exists. Returning astronauts whose orientation mechanism has been distorted and patients having vestibulopathies that may well affect their orientation mechanism are expected to have longer recovery trajectories than healthy normals.

Satisfaction of hypotheses, objectives, and specific aims of original proposal.

The first three of the original aims of this project and their hypotheses were successfully accomplished and produced the three key findings mentioned above. A fourth aim was truncated due to lack of a suitable patient population. A fifth aim was consolidated with an aim in another project. The final aim, which involved development of methodology, was integrated into the first three aims.

Implications for Critical Path Risk reduction and links to health research on Earth.

The results from this project apply directly to the Critical Path Risk of impaired neuromuscular strength upon return to positive G leading to increased occurrence of falls and fractures during emergency egress and escape – a Type II risk. We have defined some situations that occur in every day locomotion in which astronauts returning from microgravity and in which patients having subtle vestibulopathies are apt to have trouble but which there are no objective measures currently available to quantify their performance. From these, we have increased our understanding of some of the fundamental processes that govern factors like gaze and head position while moving. There is now the potential for developing more meaningful and sensitive tests of balance function that can be applied to astronauts for countermeasure assessment, and to patients with balance anomalies

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I. PROJECT RESEARCH ACTIVITY

Summary of specific aims:

Specific aim 1: Uncover the parameters that govern maintenance of upright orientation and stable gaze during locomotion. This will be done by time series analysis of body segment variables (BSV), i.e., the orientation of the feet, legs, thighs, trunk, head, and eyes during linear and circular locomotion.

Specific aim 2: Characterize the dynamics of locomotion stability in response to singular perturbations of the Body Segment Variables. For example, we will use an abrupt displacement of the support surface during walking. This should give us information about dynamics of recovery to unexpected events.

Specific aim 3: Determine the effects of locomotion on dynamic visual acuity. This will be done by asking subjects to read numbers from a visual display while they walk on a linear or circular treadmill and during singular perturbations.

Specific aim 4: Develop analytical tools to discriminate between normal physiological and pathological conditions. This will be done by testing patients with well defined vestibulopathies and developing multivariate discriminant functions to classify specific vestibulopathies, and by using nonlinear stability analysis to characterize locomotion quantitatively.

Studies and Results:

During the previous grant period, we have made considerable progress in achieving the goals set forth in our original proposal. Generally, we have established an important role of vestibulo-collic and vestibulo-ocular functions in the maintenance of stability during locomotion. Specifically, we have identified head fixation distance (HFD) and gaze fixation (GFD) as important metrics for assessing vestibular performance during continuous locomotion on a linear treadmill. These parameters should play important roles in countermeasure assessment during and following exposure to microgravity. We have developed new tools to study the compensatory and orienting functions during our proposed protocols involving turning and circular locomotion. We have improved our understanding of the kinematics of human eye movements induced by the vestibular system. We have developed two new paradigms to quantify stepping and locomotion responses. We have developed several new analitical tools.

EFFECTS OF WALKING VELOCITY ON VERTICAL HEAD AND BODY MOVEMENTS DURING LOCOMOTION:

Trunk and head movements were characterized over a wide range of walking speeds to determine the relationship between stride length, stepping frequency, vertical head translation, pitch rotation of the head, and pitch trunk rotation as a function of gait velocity. At walking speeds up to 1.2 m/s there was little head pitch movement in space, and the head pitch relative to the trunk was compensatory for trunk pitch. As walking velocity increased, trunk pitch remained approximately invariant, but a significant head translation developed. This head translation induced compensatory head pitch in space, which tended to aim the head at a fixed point in front of the subject that remained

approximately invariant with regard to walking speed. The distance to this point is termed the head fixation distance (HFD). The predominant frequency of head translation and rotation was restricted to a narrow range from 1.4 Hz at 0.6 m/s to 2.5 Hz at 2.2 m/s. Within the range of 0.8 to 1.8 m/s, subjects tended to increase their stride length rather than step frequency to walk faster, maintaining the predominant frequency of head movement at close to 2.0 Hz. At walking speeds above 1.2 m/s head pitch in space was highly coherent with, and compensatory for, vertical head translation. In the range 1.2 to 1.8m/s, the power spectrum of vertical head translation was the most highly tuned, and the relationship between walking speed and head and trunk movements was the most linear. We define this as an optimal range of walking velocity with regard to head-trunk coordination. The coordination of head and trunk movement was less at walking velocities below 1.2 m/s and above 1.8 m/s. These results suggest that two mechanisms are utilized to maintain a stable head fixation distance over the optimal range of walking velocities. The relative contribution of each mechanism to head movement depends on the frequency of head movement and consequently on walking velocity. From consideration of the frequency characteristics of the compensatory head pitch, we infer that compensatory head pitch movements may be produced predominantly by the angular vestibulo-collic reflex (aVCR) at low walking speeds and by the linear vestibulo-collic reflex (lVCR) at the higher optimal speeds of walking.

EFFECTS OF VIEWING DISTANCE ON THE GENERATION OF VERTICAL EYE MOVEMENTS DURING LOCOMOTION.

Due to the striding motion of the lower body during locomotion there is substantial vertical head movement whose magnitude and frequency are sufficient to trigger the linear and angular VOR. The role of the VOR in the generation of eye movements during locomotion is not clear. In this study head vertical translation and pitch movements during locomotion were at a speed of 1.66 m/s while subjects view a target screen placed at distances ranging from 0.25-2.0 m at 0.25 m intervals. The main finding was that for near targets eye velocity was essentially in phase with head pitch velocity (phase lead of 7.1°). Eye velocity increasingly lagged head pitch as target distance increased, and was compensatory (180° phase lag) at 2.0 m. For far targets, the gain (0.8) and phase of eye *re* head pitch velocity indicated that the aVOR was generating the eye movement response. For near targets, decreasing target distance would augment the lVOR gain, and the eye velocity phase suggested that the lVOR was generating the vertical eye movement response.

INTERACTION OF THE BODY, HEAD AND EYES DURING WALKING AND TURNING.

Body, head and eye movements were studied during straight walking and while turning corners. The purpose was to determine how well the head and eyes followed the linear trajectory of the body in space and whether head orientation followed changes in the gravito-inertial acceleration vector (GIA). During straight walking, there was lateral body motion at the frequency of stepping. The GIA oscillated about the direction of heading, according to the acceleration and deceleration associated with heel strike and toe flexion, and the body yawed in concert with stepping. Despite the linear and rotatory

motions of the head and body, the head pointed along the forward motion of the body during straight walking. The head pitch/roll component compensated for vertical and horizontal acceleration of the head rather than orienting to the tilt of the GIA or anticipating it. When turning corners, subjects walked on a 50 cm radius over 2 steps or on a 200 cm radius in 5-7 steps. Maximum centripetal accelerations in sharp turns were ≈ 0.4 g, which tilted the GIA ≈ 21° with regard to the heading. This was anticipated by a roll tilt of the head of up to 8°. The eyes rolled 1-1.5° and moved down into the direction of linear acceleration during the tilts of the GIA. Yaw head deviations moved smoothly through the turn, anticipating the shift in lateral body trajectory by as much as 25°. The trunk did not anticipate the change in trajectory. Thus, in contrast to straight walking, the tilt axes of the head and the GIA tended to align during turns. Gaze was stable in space during the slow phases and jumped forward in saccades along the trajectory, leading it by larger angles when the angular velocity of turning was greater. We think that the anticipatory roll head movements during turning are likely to be utilized to overcome inertial forces that would destabilize balance during turning. The data show that compensatory eye, head and body movements stabilize gaze during straight walking, while orienting mechanisms direct the eyes, head and body to tilts of the GIA in space during turning.

IDEAL TRAJECTORY ANALYSIS (ITA).

We investigated the hypothesis that in an ambulatory task such as repeated stepping on to a 8 cm elevation, patients with vestibulopathies would have a significantly greater deviation from an ideal center of mass displacement trajectory as compared to healthy subjects. This hypothesis was based upon spectral analysis. When averaged over nine healthy and six LD subjects, the mediolateral sway spectrum for LD's showed a much larger magnitude compared to healthy subjects in the frequency range 0 - 0.6 Hz band that is below the spectral component of the stepping frequency. To this end, we developed a quantitative method to assess repeated stair stepping stability.

In both the mediolateral (ML) and anteroposterior (AP) directions, the trajectory of the subjects' center of mass (COM) was compared to an ideal trajectory. An ideal trajectory is a combination of two coupled sinusoids, which approximate the COM trajectory of healthy subjects. Two dimensionless numbers, the ML instability index (IML) and AP instability index (IAP), were calculated using the COM trajectory. These identified ideal sinusoids for each subject, with larger index values resulting from less stable performance. The COM trajectories of nine healthy subjects and six patients diagnosed with unilateral or bilateral vestibular hypofunction were analyzed. The average IML and IAP values of labyrinth disorder patients were respectively 127% and 119% greater than those of healthy subjects ($p < 0.014$ and 0.006, respectively), indicating that the ideal trajectory analysis distinguishes labyrinth disorder patients from healthy subjects. COM trajectories identify movement inefficiencies attributable to vestibulopathy but may be limited due to variable amounts of individual adaptation.

A subsequent study of 18 healthy and 12 LD subjects revealed a strong effect of age in the indices ($p<0.001$). After correction for the age effect, we showed a significant difference between groups at the $p<0.0001$ level. We also examined the correlation between the COM, which requires a complex measurement of 11 BSV's, and a single

measure of the pelvic center. The ITA estimates calculated using just the pelvic center showed very high correlation coefficients (0.98 for ML, 0.99 for AP) with the ITA estimates calculated using the more complex measurements. This means that this repeated stepping stability analysis can be implemented using a relatively simple measurement system instead of having to measure the motion of 11 body segments. This simplification will allow us to compare ITA with perturbation response trajectories during locomotion because we can easily make both measurements in the same location.

RESPONSE TO SINGLE PERTURBATIONS ON THE BALDER PLATFORM.

This sub-project was initiated from scratch during the second year of the grant in response to the first year's critical review and required substantial development time before it was possible to take experimental data. A pilot experiment was first conducted upon six healthy subjects to determine the optimal perturbation range, and to develop the protocol. Subsequent to that a complete data set has been taken on 12 healthy subjects for perturbations delivered to the right foot during steady locomotion along a straight line using the balance disturber (BALDER) platform. The mediolateral displacement of the legs and torso have an underlying periodic component that coincides with pacing. Superimposed is a large deviation to the right that occurs after the perturbation is applied. This deviation is followed by a partial recovery toward the original line of march, but typically with a small change in direction we call drift. Less obvious is a transient reduction in the separation distance between the two legs. We have made a preliminary analysis of these data by only considering the responses on a once-per-pace basis that samples the position of both feet and the sternum at the time that one foot is in its support phase and the anterioposterior position of both feet are equal. We further analyze the difference in response between successive paces in order to eliminate the slight drift mentioned above, but to still capture the dynamics of the response trajectory.

There is a large right-goingresponse that occurs at the first step after the disturbance. The trajectory subsequently crosses the baseline at the second step to show a slight underdamped response at the third step with a return to, or near, the baseline by the fourth step. The shape and number of paces needed to recover is typical for the other three perturbations. In contrast, the recovery trajectory for a pilot vestibulopathic) takes several more paces to recover. See appendix A for figures.

Interestingly, the mediolateral leg separation distances for the vestibulopathic patient are nearly identical to the mean of the normal response. The vestibulopathic subject has normal computerized dynamic posturography scores. This would indicate a fairly subtle deficit, and would also suggest that the neuromuscular components of her postural responses are normal. Thus, one conclusion is that this subject has a distortion of her orientation mechanism which does not permit her to recover her trajectory in response to a perturbation as rapidly as healthy subject can recover

DYNAMIC VISUAL ACUITY.

Although several tests for visual blurring due to retinal slip are available commercially and several others are described in the literature, only DVA meets the unique needs and constraints of the space program for post-flight testing. Further evaluative research on the underlying concept is needed to determine if the test is sensitive to rapid VOR gain changes. DVA data were collected from 10 subjects on three postoperative days (PODs) after resection of acoustic neuromas. These preliminary results show the mean percent correct DVA scores while in the head-supported seated condition (HS).

For the HS condition the Friedman analysis was significant, $\chi^2_r = 9.24$, $p < .03$. On POD 1 scores were significantly lower than those on all other available test days. For the other conditions, insufficient data are available to determine if differences occurred. These data suggest that at least this one condition of the test is sensitive to very rapid sensorimotor adaptation. In this test subjects are given the maximum possible physical support, yet they still have decrements in performance. These data suggest the occurrence of significant oscillopsia and, indeed, although subjects were tested with their usual corrective lenses, they still complained of blurred vision and severe vertigo elicited by head movements. Thus, even with the head stabilized, subjects appeared to experience significant oscillopsia on POD 1, which resolves considerably by POD 2. Wilcoxon matched pairs signed ranks tests showed no significant differences between scores on POD 2 and 3 and between pre-operative and POD 3 tests. These data suggest that for the HS condition the test is sensitive to the type of rapid change caused by the surgery and the resulting compensatory processes that are elicited during the most acute phase of recovery. Unfortunately, the number of operated patients available to the collaborating otologist at Baylor College of Medicine dramatically decreased during the second year. Thus, this sub-project was not continued into year three.

ROBUST PUPIL CENTER DETECTION USING A CURVATURE ALGORITHM.

We have developed a new algorithm that utilizes curvature characteristics of the pupil boundary to eliminate artifacts due to eyelids, eyelashes, corneal reflection and shadows. Pupil center is computed based solely on points related to the pupil boundary. For each boundary point, a curvature value is computed. Occlusion of the boundary induces characteristic peaks in the curvature function. Curvature values for normal pupil sizes were determined and a threshold was found which together with heuristics determined normal from abnormal curvature. Remaining boundary points were fit with an ellipse using a least squares error criterion. The center of the ellipse is an estimate of the pupil center. This technique is robust and accurately estimates pupil center with less than 40% of the pupil boundary points visible.

VALIDATION OF FLOQUET ANALYSIS.

Before reporting on transients of gait initiation during treadmill walking, we decided to validate the Floquet multiplier method originally proposed. We used

simulated data having known dynamics like a decaying exponential, a cosine modulation, and their product: an exponentially decaying sinusoid. From these simple models one can calculate the theoretical eigenvalues and then compare them with the results obtained from the original Floquet multiplier method. This approach produced mixed results. A first order system with no noise yielded multiplier estimates that agreed with the theoretical values. Higher order systems had multipliers that were somewhat related to theory but which did not completely identify the roots of the model system. The original method sometimes yielded a real root that was approximately equal to the magnitude of the largest theoretical root. When we added noise to the model the situation was further confounded by the appearance of yet another multiplier. Sometimes this multiplier was larger than the ones we wanted to identify. We have since applied a new method called generalized Floquet theory (GFT) that can identify systems of higher order than two: a limitation of the original. GFT uses time shifted "pseudo states" (more than one sample per cycle) that are analogous to the use of higher derivatives. GFT has also been validated and works well for systems of order 4 and less when there is little or no noise. GFT analysis of treadmill gait initiation showed significant differences between trials when the subject jumped onto the already moving treadmill compared to trials when the subject walked to a ramped start-up. The original analysis did not show this difference. Thus, the GFT analysis is now validated for use in further studies, for example the response to perturbations of gait, specific aim 1.

Significance

The significance of the studies over the last grant period is that we have gained considerable insight into the compensatory and orienting functions of the vestibulo-collic and vestibulo-ocular reflexes during locomotion. Through this has come the development of countermeasure assessment criteria which can now be applied in studying behaviors that have proven to be difficult following exposure to microgravity. The perturbation studies have produced a simple, easy-to-implement technique of quantitative gait assessment during repeated stepping, and a novel paradigm for studying the responses to precisely applied gait disturbances which simulate a slip during locomotion.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH

Plans

Over the next grant period, we intend to extend our objective assessment paradigms to include the most important locomotor activities of making turns, and walking up and down a flight of steps. This will allow us to address the Type II Critical Path Risk: Impaired neuromuscular strength upon return to positive G leading to increased occurrence of falls and fractures during emergency egress and escape.

A parametric analysis will be developed for the responses to perturbation during locomotion to allow for simple inter- and intra-subject comparisons. . By comparing the existing data from normals with the data of vestibulopathic subjects taken over the first year of the proposed grant, we will develop a quantitative, parametric approach for

establishing the limits needed to apply this paradigm for detecting subtle deficits to disturbances of locomotion.

The repeated stepping tests developed during the last grant period will be compared with the new parametric gait perturbation analysis. In addition, we plan to evaluate the potential of two measures designed to counter the imbalance caused by distortions in the orientation mechanism due to microgravity exposure which degrades stability during locomotion.

Appendix A. Project Research Data (selected)

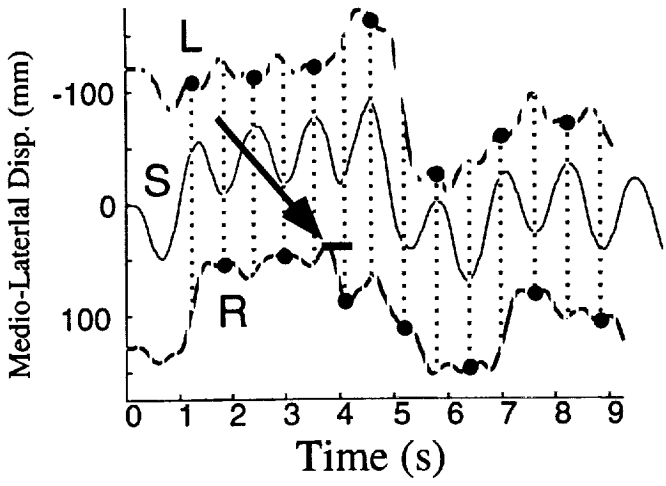


Fig. SA1-1. *Mediolateral leg and torso displacements for a 10 cm forward-right perturbation delivered to the right foot during steady locomotion. Arrow shows direction of applied disturbance, while horizontal bar shows its duration. Solid circles show locations of the stance foot at times when both legs have same anteroposterior position. These events are the samples that are used for subsequent analysis.*

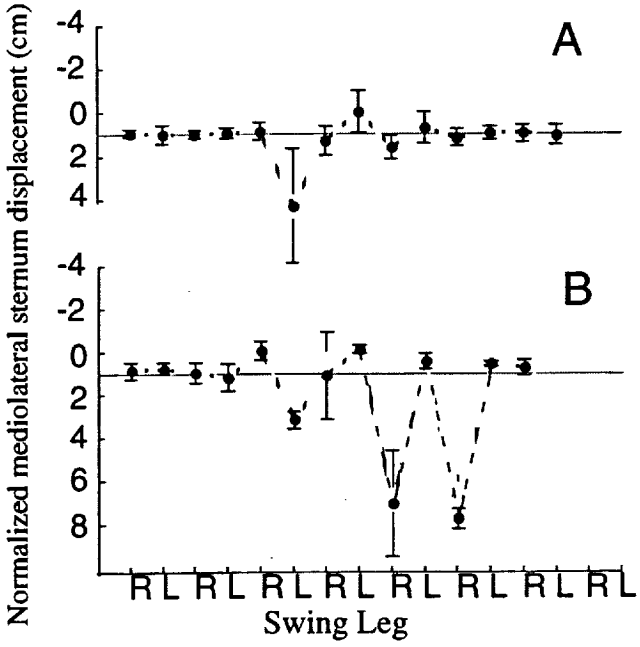


Fig. SA1-2. *A) Mean normalized, differenced mediolateral sternum displacement for a group of 12 healthy subjects in response to a 10 cm forward-right perturbation delivered to the foot. Errorbars mark ± 1 standard error. Recovery to baseline is in three paces. B) Response of vestibulopathic subject to same perturbation. Recovery to baseline is in about five paces.*

Appendix B.

TITLES AND COMPLETE REFERENCES TO ALL PUBLICATIONS AND MANUSCRIPTS SUBMITTED OR ACCEPTED (10/1/97 – 9/30/2000):

During the grant period, there were 8 refereed publications related to this grant. There was also a symposium on locomotion led by Dr. Raphan held at the Neural Control of Movement (NCM) in April, 1999. In addition there were a total of 11 abstracts at neuroscience (3) and the Head and neck Symposium in Japan(4), and four other abstracts.

PAPERS:

1. Imai, T. Moore, S. T., Raphan, T. Cohen, B. Interaction of the body, head and eyes during walking and turning. Exp. Brain Res. (In Review, 2000)
2. Thurtell, M. J., Kunin, M., Raphan, T. Role of muscle pulleys in producing eye position-dependence in the angular vestibulo-ocular reflex: A model based approach, J. Neurophysiol. (In Press, 2000)
3. Moore S.T., Hirasaki E., Cohen B. and Raphan T. Effects of viewing distance on the generation of vertical eye movements during locomotion. Exp. Brain Res. 129:347-361.
4. Hirasaki, E., Moore, S.T., Raphan, T., Cohen, B. Effects of walking velocity on vertical head and body movements during locomotion. Exp. Brain Res. 127: 117-130, 1999.
5. Zhu, D., Moore, S.T., Raphan, T. Robust pupil center detection using a curvature algorithm. Computer Methods and Programs in Biomedicine, 59 (3): 145-157, 1999.
6. M.D. McPartland, D. E. Krebs, C. Wall III. Quantifying Ataxia: Ideal Trajectory Analysis, J Rehabil Res Develop, accepted for publication.
7. D.J. Newman, R. Wu, D. Krebs, D.K. Jackson. Electromyographic analysis of human false platform jumping. In revision, J. Appl. Physiol.
8. McPartland MD, Wall C, Oddsson LI, Krebs DE, Tucker CA. Recovery from perturbations during paced walking. Submitted to Gait and Posture.
9. Dimitri PS, Wall C, Oas JG and Rauch SD. Application of multivariate statistics to vestibular testing: discriminating between Meniere's disease and migraine associated dizziness. Accepted with revisions to J. Vestibular Research.

Symposium:

1. Raphan, T. Body, head and eye movements during locomotion: New turns. Panel, Neural Control of Movement, Participants: T. Raphan, J. Demer, Aftab Patla, and J. Bloomberg. April 12, 1999

Abstracts:

1. Imai, T., Moore, S.T., Raphan, T., Cohen, B. Posture and gaze during circular locomotion Soc. Neurosc. Abstr. (2000).
2. Raphan, T., Imai, T., Moore, S.T., Hirasaki, E. and Cohen B. Quantitative representations for analyzing and modeling 3-D body and head movements during

locomotion. The IVth International Symposium on the Head/Neck System, Japan, 1999.

3. Moore, S. T., Hirasaki, E., Raphan, T. Cohen, B. Effects of viewing distance on head-eye coordination during locomotion. The IVth International Symposium on the Head/Neck System, Japan, 1999.
4. Hirasaki E., Moore S.T., Raphan T., Weinberg J. and Cohen B. Head movements during circular locomotion in normal subjects. Proc. Soc. Neurosc. (62.12) 24: 153, 1998.
5. Imai T., Hirasaki E., Cohen B. and Raphan T. Stabilization of gaze when turning corners during overground walking. Soc. Neurosc. (162.5) 24: 415, 1998.
6. Moore S.T., Hirasaki E., Cohen B. and Raphan T. Generation of vertical eye movements by the VOR during locomotion. Soc. Neurosc. Abstr. (651.15) 24: 1659, 1998.
7. Wall C, Krebs DE. Application of floquet stability analysis to repeated stepping in LD and normal subjects. First Biennial Space Biomedical Investigator's Workshop. League City, TX Jan 11-13, 1999.
8. McPartland MD, D. E. Krebs, C. Wall III. Quantifying Instability During Stepping via Ideal Trajectory Analysis., 1999 Annual Fall meeting of the Biomedical Engineering Society and the 21st Annual International Conference of the IEEE Engineering in Medicine and Biology Society in Atlanta, Georgia, Oct 13-16
9. Sensitivity of the Dynamic Visual Acuity test to sensorimotor change. 1999 Cohen, H., Bloomberg, J.,Elizalde, E., Fregia, M. Proc.Houston Society for Engineering in Medicine and Biology
10. Cohen H, Blomberg JJ. Dynamic visual acuity tests in acoustic neuroma patients, Midwinter meeting of the Association for Research in Otolaryngology, St. Petersburg Beach, FL Feb. 20-24, 2000
11. Wall C, McPartland MD, Raphan T. Human gait stability in response to perturbations duirng treadmill walking. Midwinter meeting of the Association for Research in Otolaryngology, St. Petersburg Beach, FL Feb. 20-24, 2000

NSBRI RESEARCH PROGRAM
RADIATION EFFECTS

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Yang, T.	CO-I	NASA JSC (Deceased)	
Huso, D.L.	**PI**	**Hopkins/SOM**	**Chemoprevention of Radiation-Induced Rat Mammary Neoplasms**
Dicello, J. F.	CO-I	Hopkins/SOM	
Williams, J. R.	**PI**	**Hopkins/SOM**	**Radiation-Induced Cytogenetic Damage as a Predictor of Cancer Risk for Protons and Fe Ions**
Griffin, C. A.	CO-I	Hopkins/SOM	
Zhang, Y.	CO-I	Hopkins/SOM	
Sinden, R. R.	**PI**	**Texas A&M**	**Quantitation of Radiation-Induced Deletion and Recombination Events Associated with Repeated DNA Sequences**
Braby, L. A.	CO-I	Texas A&M	

National Space Biomedical Research Institute

FINAL PROGRAM REPORT

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The information in this report is preliminary, proprietary, and confidential

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COVER PAGES

National Space Biomedical Research Institute

Radiation-Effects Team

TEAM PROJECTS AND PRINCIPAL INVESTIGATORS

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PROGRAM EXECUTIVE SUMMARY

Radiations in space from galactic and solar sources are generally considered to be one of the three or four most serious hazards with regard to long-term human missions. A recent National Research Council/National Academy of Sciences (1996, 1998) report, for example, and an earlier related report (1996) discuss the significance and consequences of radiation exposures in space. The NASA Critical Path Roadmap (http://criticalpath.jsc.nasa.gov/main.asp) classifies Radiation Effects as one of four Type-I severe risks, those of most concern, along with Bone Loss, Human Behavior, and Clinical Capability. NASA's Critical Path Roadmap lists five major risks from radiation, categorizing them with "I" being the most significant risk type and "1" being the most significant risk rank:

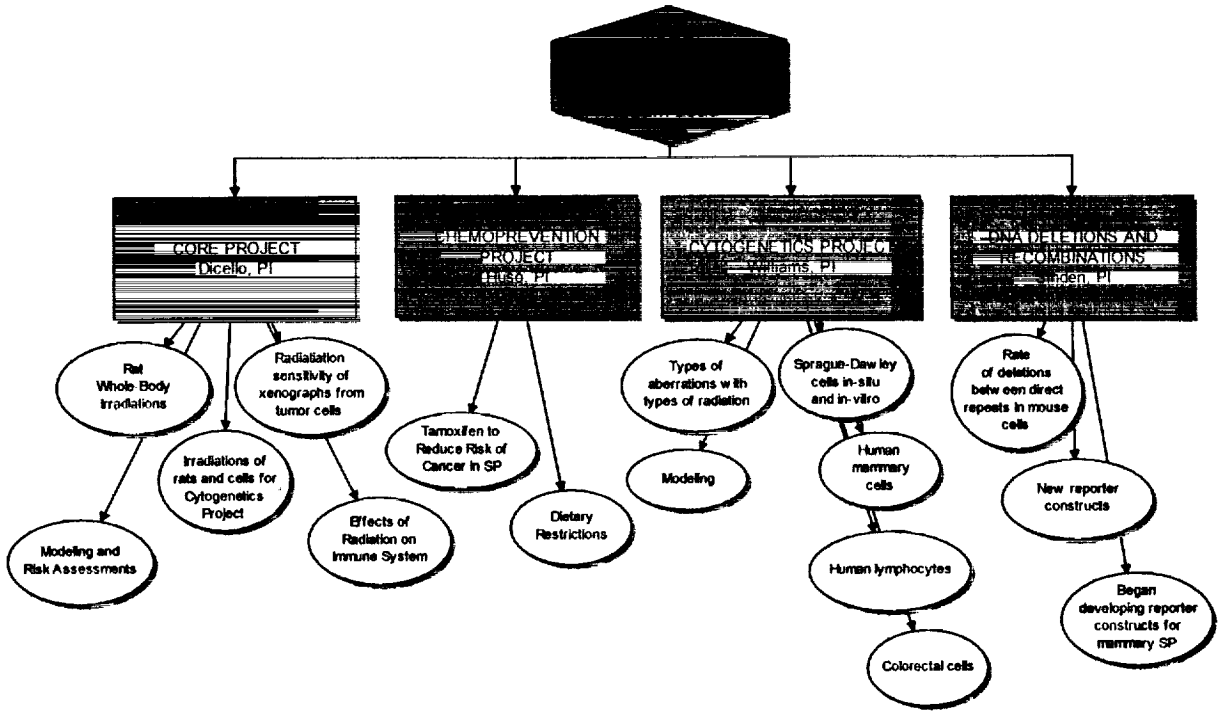
Table 1. Potential consequences from radiation exposures in Space, according to the NASA Critical Path Roadmap.

Risk Factors	Risk Type	Risk Rank
Carcinogenesis	I	1
Damage to the central nervous system from radiation	II	2
Synergistic effects from radiation, microgravity, and other spacecraft environmental factors	II	3
Early and acute effects from radiation exposure	II	4
Radiation effects on fertility, sterility, and heredity	III	5

The National Space Biomedical Biomedical Research Institute (NSBRI) was formed to address the medical risks to humans in space as well as the subsequent risk to mission success. One of NASA's goals in supporting the institute is to understand the effects of space radiation on biological, chemical, and physical systems and processes. An essential strategy of the NSBRI has been the Integrated Research Team concept to maximized productivity and cost-efficiency with minimum redundancy to build and balance integrated research programs. A main mission of the NSBRI is to design and validate countermeasures addressing the major hazard. At the end of the first three years, the Radiation-Effects Team has become one of the most productive, cohesive teams focusing on the first four risk factors in Table 1 and one of the few to have shown feasibility of a proposed countermeasure, the use of chemopreventive agents or dietary supplements to reduce the risk of cancer from low-dose exposes to high atomic-number, energetic charged particles (HZEs) and protons.

The present Team organization, as outlined in the following figure, evolved from the original missions of the NSBRI and the Phase-I and Phase-II NSBRI proposals to NASA. The overall philosophy is that the Core Project provides the *in-vivo* results for risks of cancer and other diseases and the Chemoprevention Project provides the data for risk reduction with Tamoxifen. The Cytogenetics Project gives us cellular and cytogenetic data for chromosome aberrations for cells irradiated in the animal or in-vitro and the DNA Project provides us with mutation data related to repeated sequences, in both cases to provide us mechanistic information for extrapolation to humans and parametric data and benchmarks for risk assessments. Finally, the Core Project assembles all of the data to calculate risks in the animal model and to extrapolate to risks of humans in space.

Research Structure for the Radiation Effects Team



An outline of the organizational structure and research accomplishments of the Radiation-Effects Team.

Almost every review of the radiation problems in space, including the three previous references, has recommended animal studies to quantify the risks to these types of radiation and to pursue likely countermeasures. Until this series of experiments, however, there had been only one comprehensive animal study to investigate the effects of ions of high atomic number and high energy, HZEs. That experiment was conducted by Alpen et al. (1993) with the Berkeley Bevalac, which has been out of commission for almost a decade. It has provided invaluable data on carcinogenesis in the harderian gland of a mouse model as a function of linear energy transfer (LET), and it has been a cornerstone for risk assessments in space during the last decade. No comparable series of experiments had been conducted to evaluate the use of drugs to reduce the risk of cancer from exposures in space.

As a result of the scientific reviews, meetings, and discussions during the developmental period of the NSBRI, the Core Project chose as its animal model the female Sprague-Dawley rat to be irradiated whole-body with HZEs, protons, or photons, and evaluated the biological consequences, including malignant and benign tumors at all sites, pituitary and CNS damage, and other diseases. The motives for this choice were multiple and are presented in detail in the Final Report for the Core Project; however, this model was that recommended by the members of the External Advisory Council. Animal experiments of this type were generally not done previously because of the complicated logistics and the large expensive.. Only three facilities in the world, one at Brookhaven National Laboratory in New York State, one in Germany, and one in Japan, produced the necessary accelerator HZE beams. The costs for HZE beam time is millions of dollars a year, far in excess of any funds available from the NSBRI, and only about 150 hours a year are available for all space-biology irradiations in the U.S.A. Finally, no one had ever carrying out experiments with energetic charged particles at multiple facilities , including Loma Linda University with its energetic proton synchrotron.. The logistics







of transporting thousands of animals between multiple facilities in isolated environments and keeping them alive subsequently for three or more years was at best a challenge. To maximize the value of the results, the team lead offered colleagues in the other projects to join forces to maximize the production of useful scientific data with the irradiated animals and to provide different information but correlated to the same animal species and to humans so that the results could be applied most efficiently to humans in the space environment. Three other projects that successfully survived the review process with two joining forces to use the Sprague-Dawley, one studying Tamoxifen (Howard/Huso) as a chemopreventing agent and the other to look at cytogenetics to predict cancer risks (Williams) and to correlate the Spraque-Dawley results to human mammary cells, human lymphocytes, and eight different human colorectal cell lines with varying status of p53 expression. The third project (Sinden) proposed originally to use low-energy helium microbeams to study repeated DNA sequences using techniques that were established at that time only for mouse models. During the intervening time, the principal investigator has redirected the project goals to study the more energetic iron beam at BNL and has been developing reporter constructs for the Spraque-Dawley rat.

At the end of the first funding cycle, the four projects have become almost indistinguishable in their goals and in their cooperation. That focused, cooperative effort has succeeded in implementation and execution of one of the most relevant but most difficult series of experiments performed as part of NASA's Life Sciences program in at least a decade.

At the end of the first three years of the team program, the investigators have designed, built, and successfully implemented systems to transport and irradiate both animals and cells. Three series of experiments were performed, each examining the consequences of 1-GeV iron ions, 250-MeV protons, and gamma rays from cesium-137 and cobalt-60, as well as sham irradiations. In each case, the animals were irradiated whole body at doses comparable to those expected in space. The animals are cared for and monitored daily, and all diseases are medically treated.

We now have statistically siginificant data for the risk for mammary fibroadenomas and adenocarinomas and for pituitary-related diseases as functions of particle type, dose, and time, as discussed at length in the project reports. In parallel with the animal experiments, we have been examining cell systems. The principal objectives of these projects were to examine cell survival, cytogenetic damage, and DNA deletions and recombinations understand the initial damage and the mechanisms responsible for the initial damage and the subsequent promotion and progression of the diseases. We are developing theoretical models to simulate the the biological alterations and the in-vivo responses. We have used our data and models for preliminary calculations for the risk of cacinogenesis in the animals.

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I. PROGRAM RESEARCH ACCOMPLISHMENTS

A. Team Goals and Strategy

The research strategy detailed in the original Phase-II proposal for the Radiation-Effects Team was:

1. To design protocols for exposing animal models to accelerator-based high-energy proton and heavy-ion (HZE) beams, including detailed biostatistics of numbers of animals exposed, the protocol, and the expected level of certainties.

Completed

2. To design experimental procedures and to build the equipment and apparatus necessary to carry out the irradiations and to do the dosimetry and physics to characterize the irradiations.

Completed

3. To irradiate rat mammary glands, in vivo, and both rat and human mammary epithelial cells in vitro with photons, protons, and, initially, iron ions to evaluate tumor formation in rat mammary glands with and without chemoprevention.

Completed.

4. To evaluate response to rat mammary epithelial cells irradiated in vitro and in vivo and human epithelial cells irradiated in vitro with photons, protons, and iron ions and compare these responses with other cell types.

Completed.

5. To irradiate mouse lungs in vivo with photons, protons, and initially, iron ions in order to evaluate tumor formation in mouse lungs with and without chemoprevention.

This proposal was not funded.

6. To irradiate the same animal models with both energetic protons and iron, to evaluate the level of (predicted) synergistic, i.e., non-additive, effects of sequential or simultaneous irradiations, more closely approximating the space environment.

This strategic goal was eliminated prior to initiation of the project because of reduced funding and the complex logistics of irradiating the same animals at widely separated facilities. Nevertheless, a preliminary experiment was successfully executed and the results are presented below.

7. To examine the changes in response with fractionation and changes in dose-rate.

This strategic goal also was eliminated prior to initiation of the project because of reduced funding. Again, a preliminary experiment was successfully executed and the results are presented subsequently.

8. To model the biological results, so the data may be extrapolated to humans for the scenarios expected in space.

We are developing a new, non-linear model which we are successfully applying to our cytogenetic data and our animal model to calculate the risk of carcinoma.

9. To establish risks relative to low-LET radiations.

We have been careful that all of our experiments have had both unirradiated shams and photon-irradiated animals.

10. To establish safe pharmaceutical countermeasures to those risks.

With establishing the magnitude of the health risks from the radiation exposures, this is our most important goal. Although we expected no definitive results until about five years into the study, we are able to report that our initial results show that the risk of mammary cancer arising from exposures of our animal model to HZE particles is definitely reduced.

B. Other Goals and Strategies

The original consortium that wrote the Phase-I proposal and the first research priorities for the NSBRI consisted of six institutions. Texas A&M University was added as a consortium institution prior to the submission of the Phase-II proposal. Dr. Richard Sinden from that institution submitted a research proposal, "Quantification of Radiation Induced Deletion and Recombination Events Associated with Repeat DNA Sequences," which did well in the scientific review, and the proposal received funding, although a reduced level even greater than that for the other proposals. Although the proposal was outside of the original strategy for the Radiation-Effects Team, Dr. Sinden, the Team Lead, and the Team investigators worked together successfully to incorporate this research into the overall Team Strategy, as discuss later. The project's initial specific aims were:

a. To quantitate deletion between direct repeats in progeny of cells exposed to high LET radiation and simulated delta rays.

Modified to align with Team Strategy and to account for reduced funding.

b. To quantitate deletion between direct repeats in cells adjacent to those irradiated by high LET particles and or simulated delta rays.

Modified to align with Team Strategy and to account for reduced funding.

c. To quantitiate deletion between direct repeats in cells adjacent to those irradiated by high LET particles and or simulated delta rays, i.e., in "bystander cells."

Not done because of reduced funding.

d. To develop additional reporter constructs to increase the spectrum of mutational events that can be quantitated. Specifically, constructs that report deletion, recombination, and gene conversion events will be developed.

Modified to align with Team Strategy and to account for reduced funding.

C. Accomplishments

1. To design protocols for exposing animal models to accelerator-based high-energy proton and heavy-ion (HZE) beams, including detailed biostatistics of numbers of animals exposed, the protocol, and the expected level of certainties. **Completed (Core Project).**

All of the biostatistical and epidemiological aspects of the experiments were designed and periodically reviewed and updated in consultation and collaboration with Dr. Steven Piantadosi, Director of Biostatistics of the Johns Hopkins Oncology Center. Much of the theoretical and statistical analyses are described in some detail in the final reports for the individual projects and summarized in this report. The successful outcomes of essentially every planned experiment in terms of selecting the appropriate dose ranges obtaining relevant responses and selecting the appropriate numbers of animal for sufficient sensitivity and certainty attest to the success of the initial planning in this area.

Large accelerator facilities, including those at LLUMC and BNL, investigators are required to write protocols and supporting documentation for experiments and submit them for approval to the appropriate animal use and program advisory committees, and all experimentalists, including students and technicians, and generally are required to successfully complete special courses and pass written examinations yearly.

Proposals or protocols have been submitted initially and yearly to:

a) Johns Hopkins protocol committees,
b) Brookhaven National Laboratory (BNL) Committees (protocol and program advisory committees),
c) Loma Linda University Medical Center (LLUMC) Committees (protocol and program advisory committees.

Proposals and protocols are routinely submitted and reviewed for :

a) Spraque-Dawley (SP) rat,
b) nude mice (for xenographs of SP cells),
c) human cells.

All necessary submittals have successfully resulted in generating approval for the research, and all researchers have successfully completed the necessary instructional work and have passed all necessary examinations.

2. To design experimental procedures and to build the equipment and apparatuses necessary to carry out the irradiations and to do the dosimetry and physics to characterize the irradiations. **Completed (Core Project).**



Fig. 1. One of the systems for irradiating animals at the Brookhaven National Laboratory.

Because of the unusual environments under which these experiments are being carried out and unique difficulties associated with accessing the irradiation areas, special apparatuses had to be designed and constructed for these experiments. Customized holders and irradiating set ups were designed and built for rats, 25-ml flasks, 5-ml test tubes, and 15-ml test tubes were designed for the irradiations. However, the restraining devices were standard commercial devices to ensure the animal welfare.



Fig. 2. One of the apparatuses for irradiating cells at the Loma Linda University Medical Center.

Because secondary particles produced as the primaries traverse the samples can be a major factor in determining response, each apparatus was designed so that the samples could be completely surrounded by absorbing Lucite, chosen because of it close approximation of muscle tissues for HZEs and because of its ability to be machined into intricate structures. A variable amount of absorber could be added







upstream and/or downstream of the biological sample, and the samples could be inserted and removed from the beam in a rapid manner. Dr. Jack Miller, Lawrence Berkeley Laboratory, and his colleagues were ableto work with us to develop a special iron beam with about 10% uniformity over more than a 10-cm diameter. At LLUMC, Dr. Michael Moyers work with us to produce a comparable proton beam. Beam parameters and characteristics were particularly crucial for the rat irradiations where we were doing total body irradiations. With several hundred rats to irradiate and with access through the safety interlocks and doors to the beam lines taking several minutes, efficiency was a major consideration as well. The final apparatuses used successfully at our first and subsequent experiments with 1-GeV iron ions at Brookhaven and 250-MeV proton beams at Loma Linda University Medical Center are shown in Figures 1 and 2.

3. To irradiate rat mammary glands, in vivo, and both rat and human mammary epithelial cells in vitro with photons, protons, and, initially, iron ions to evaluate tumor formation in rat mammary glands with and without chemoprevention. **Completed.**

i) Tumor formation in the Sprague-Dawley rat after irradiation with iron, protons, cesium or cobalt gammas (Core Project)

Approximately three thousand rats have been irradiated with 1-GeV iron ions, 250-MeV protons, and cesium-137 and cobalt-60 gamma rays. Doses for the protons and photons typically ranged between 50 and 500 cGy, and the doses for the iron beams typically ranged between 5 and 50 cGy. Some higher doses were used in the beginning of the experiments because the Relative Biological Effectiveness (RBEs) for the protons and iron ions had not been measured previously, for in the case of the Tamoxifen studies were there is a reduction in the levels of tumor induction with the administration of the drug. Details of the number of animals at each specified dose are delineated in the final report for the Core Project.

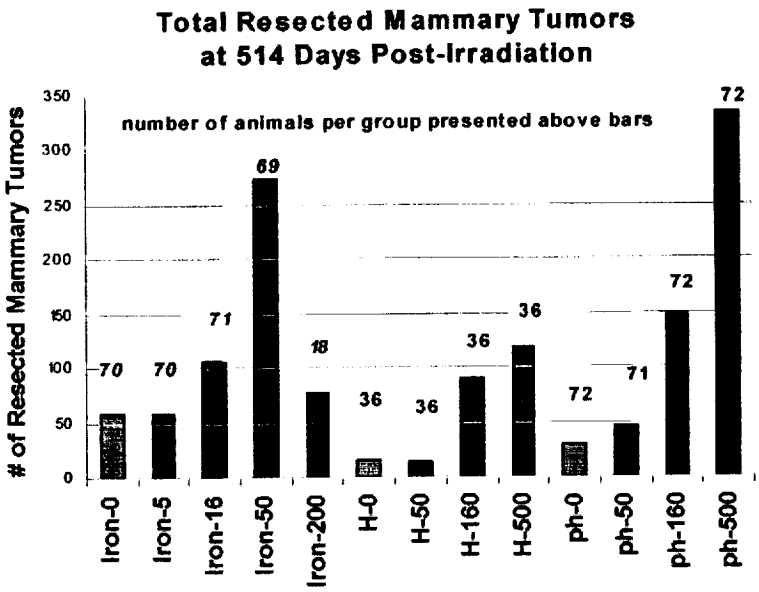


Fig. 3. The number of excised tumors observed as a function of radiation type and dose.

Tumors have been observed in the primarily in the breast and thyroid, but also to a lesser extent in the thyroid and other sites. The Sprague-Dawley rat has a high natural incidence of mammary fibroadenomas and adenocarcinomas; however, with the excision of mammary tumors at a relatively modest size, the the major cause of death for our colonies is pituitary-related illnesses. Although an examination of pituitary diseases was not a specific aim of these projects, we have been examining theses tissues and analyzing the results with internal resources, and these results will be published when they are complete..

Figure 3 shows the number of mammary tumors resected, as a function of dose and radiation type. The number of tumors increases with increasing dose of a radiation type. The small count of Iron-200cGy tumors is a reflection of the reduced number of animals in that group. Slightly less than half of all

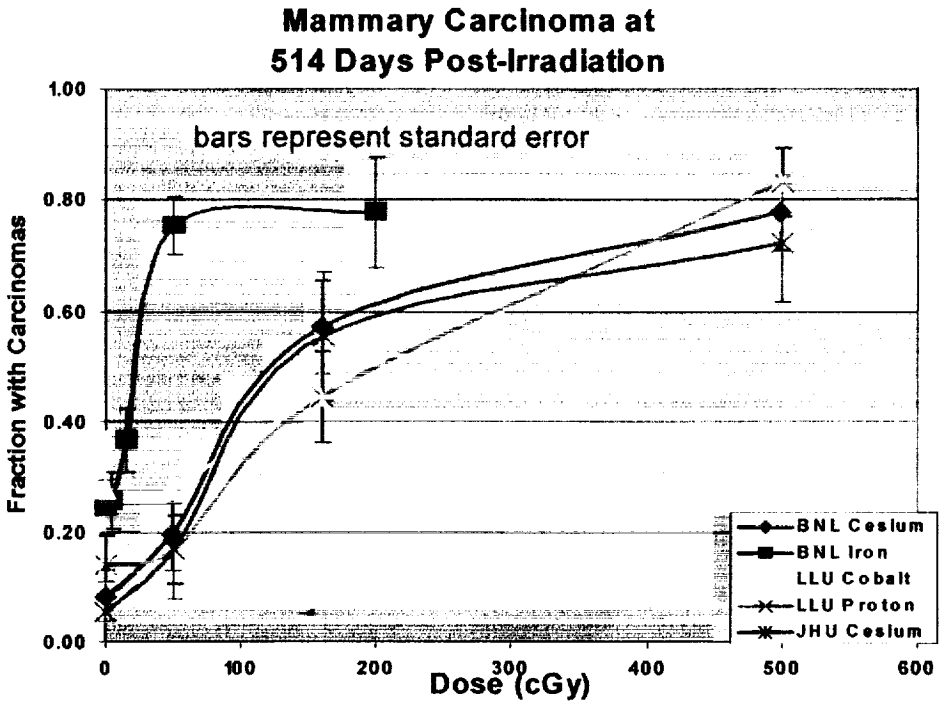


Fig. 4. Fraction of mammary carcinomas observed in each group at 514 days after irradiaton.

radiation dose/type group with a resected mammary carcinoma is shown in Figure 4. A statistical analysis of proportions indicates there is no difference between each individual sham group; therefore, at this point, they are being pooled for subsequent analyses. The group of rats irradiated with Cesium at BNL, those irradiated with Cesium at JHU, and those irradiated with Cobalt at LLUMC were compared within each dose level and also found to be statistically similar and therefore pooled into single photon groups at each dose to increase statistical power. Analyses demonstrate that iron-5cGy (p=0.14) is not statistically different from the shams at this time. The proton-50cGy (p=0.91) or photon-50cGy (p=0.53) group histopathologically classified mammary tumors are carcinomas. There does not appear to be a strong trend in the carcinoma/fibroadenoma ratio with dose or radiation type. It should be noted that each animal has 12 mammary glands, each of which can develop one or more tumors. Thus the same number of tumors in groups of the same size, such as shams and low dose groups, does not mean the same number of animals with tumors.

The fraction of animals in each

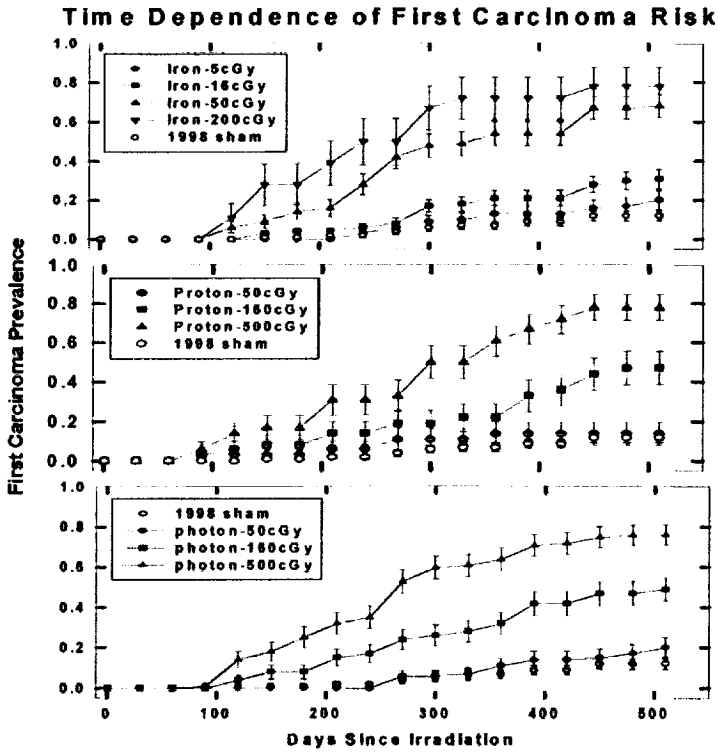


Fig. 5. Prevalence of first carcinoma as a function of time after irradiation.

responses are also not different from the shams at 514 days post-irradiation. The higher dose groups of each radiation type are significantly different ($p<0.05$) from the shams. Within the proton and photon irradiated animals, the response from each dose group is unique.

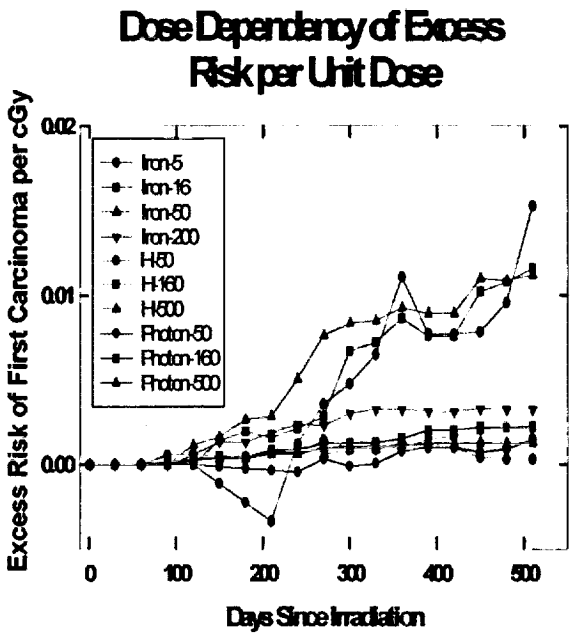


Fig. 6. Excess risk per unit dose.

Prevalence of first carcinoma as a function of time after irradiation is presented in Figure 5 for each radiation group. The top graph illustrates first carcinoma response of iron-irradiated animals versus shams. A paired sample t-test was conducted and all curves for iron irradiation were significantly different from the shams except for iron-5cGy ($p=0.54$). Similar analyses were performed on the proton and photon curves within each radiation type. It was found that all curves differed significantly from the sham curves, including the curves for the lowest doses of protons and photons. When comparing the proton curve versus the photon curve at the same dose, it was found that only the highest dose of 500cGy resulted in statistically similar curves. This may be significant in that it is frequently assumed in risk assessment that the carcinogenic effects of protons and photons are similar at identical doses.

To evaluate the linearity of effect per unit dose, excess risk of a first carcinoma per cGy was plotted for each dose group in Figure 6. As expected, the effect per unit dose is greater for the iron ions than the protons or photons. The notable exception is the iron-200cGy group in which the excess risk is diminished. This effect is likely caused by the fact that, at this high dose, few animals remain without a carcinoma, so the pool of susceptible animals is diminished. Again, because we did not know responses, we chose doses that would likely bracket the regions of interest. The tight grouping of the 5cGy, 16cGy and 50cGy iron groups is one indication that we are within the linear region of the iron dose scale, at least up to 514 days post-irradiation. At 514 days, RBEs between 1 and 10 appear consistent with the data, depending upon the time and dose-region of interest.

In an effort to correct our analyses for the number of animals at risk at any given time, a Kaplan-Meier survival analysis was performed (Figure 7). A Mantel-Haenszel log rank test of KM survival estimators indicates no difference between shams and iron-5cGy ($p=0.07$). The lowest-dose proton and photon groups are also not significantly different from the sham response. A comparison between proton and photon groups at the same dose level shows no significant difference.

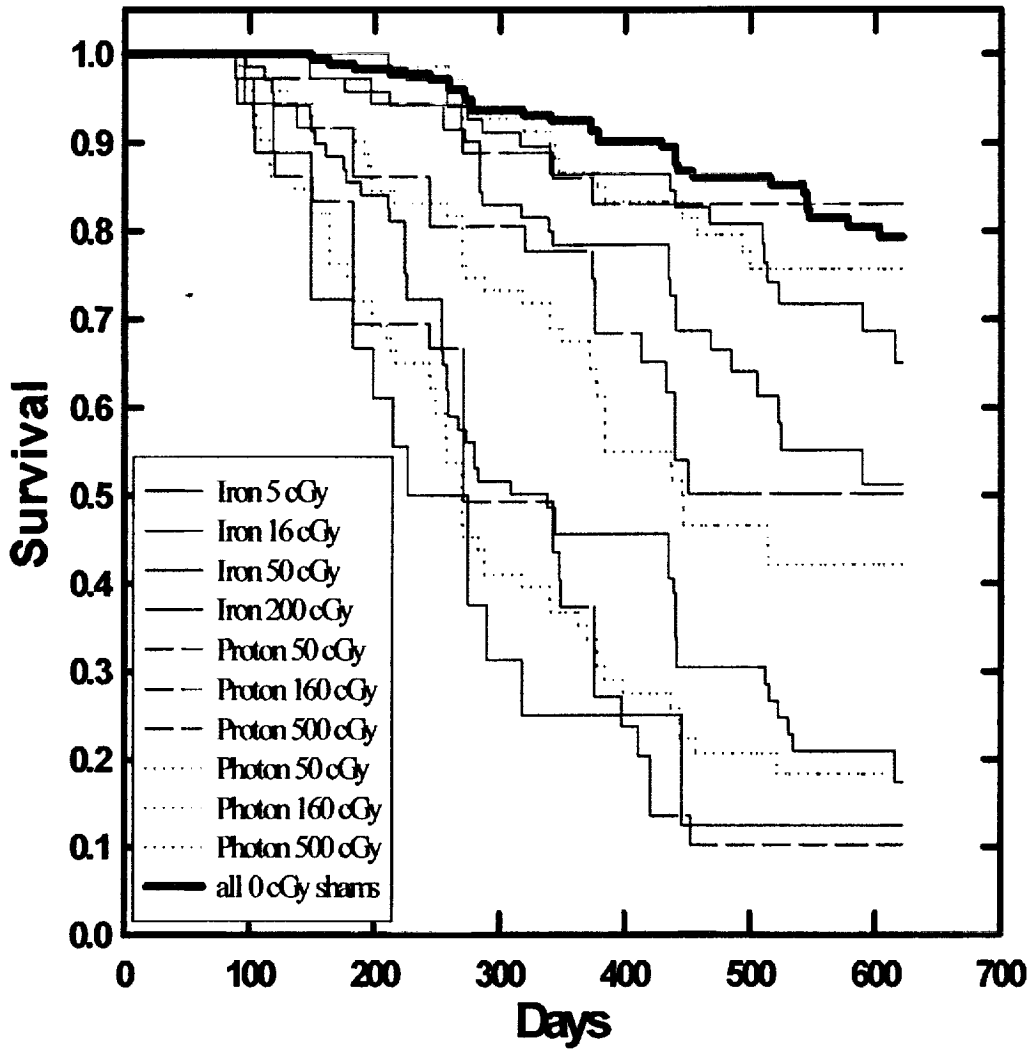


Fig. 7. Results of a Kaplan-Meier survival analysis.

The relative risk (RR) of a first carcinoma (in reference to the sham group) versus time post-irradiation is plotted in Figure 8. The relative risks integrated with time demonstrate the strong dose dependence of time to first carcinoma. The fact that the RR is decreasing with time is an indication that the sham animals are developing mammary carcinomas at a greater relative rate that the highest dose groups. The near linearity of the moderate and low dose groups through time may be interpreted as support that the

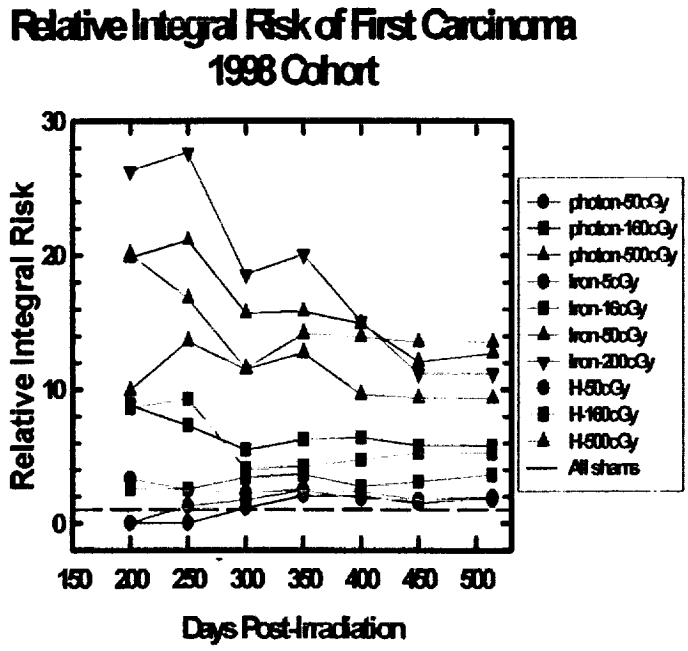


Fig. 8. Relative risk as a function of time since irradiation.

relative rate of cancer development is similar to that in the sham group. Such a study of the time-dependence of risk is not possible with most in-vivo animal studies as they typically utilize prevalence at the end of study as the major endpoint.

Figure 9 illustrates the effect of using an additional 11.5 cm lucite shield when irradiating the animals with iron ions. This amount of lucite reduces the average energy of the beam to about 600 MeV/amu, a typical beam energy used previously at the Berkeley Bevalac. Although the spectrum of radiation present behind the Lucite is of a lower energy and lower atomic mass and has a different lineal energy distribution, no measurable difference in carcinogenic effect is seen.

ii) Tumor formation in the Sprague-Dawley rat after irradiation without and with Tamoxifen (Chemoprevention Project)

The chemoprevention study control group is of particular interest because they receive up to 25% dietary restriction to match their weight gains to the corresponding tamoxifen treatment groups. Dietary caloric restriction has been shown to significantly improve the longevity of rats. Therefore in this group a higher percentage of animals should survive to old age and overall they likely will live longer. The total number of rats that have been enrolled in this group, receiving no tamoxifen, is 915. More than 90% of these are still being palpated and tumors biopsied. Lifetime analysis of these will be extremely useful for

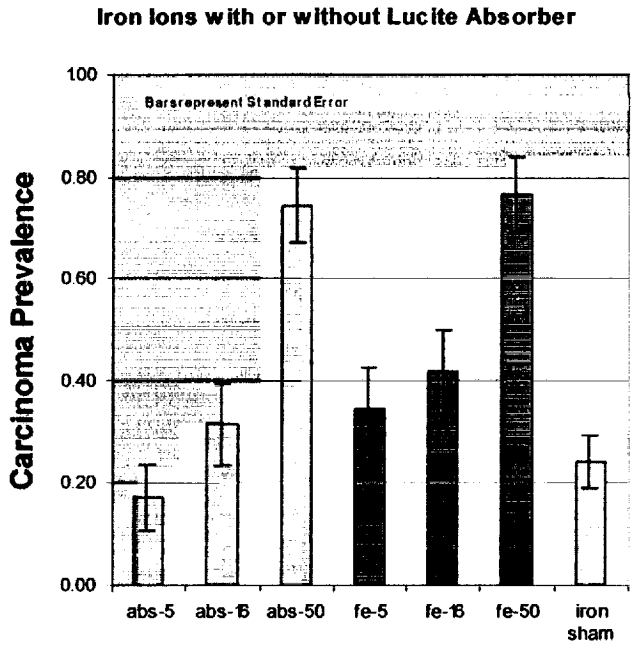


Fig. 9. A comparison of prevalence with lucite placed upstream of the rat during irradiation.

determining the radiation effects of protons and iron ions, especially at low dose exposures.

The total number of rats that have been enrolled as irradiated animals receiving Tamoxifen chemoprevention is approximately 915 also. More than 90% of these are still alive. This cohort is proving extremely valuable in determining the effectiveness of tamoxifen administration as a chemopreventative against the most relevant mammary tumors likely to be encountered following low-dose proton or iron ion exposure including those that occur later in life. The Tamoxifen Chemoprevention Study of group of rats receive continuous tamoxifen through an implanted slow-release pellet and they do not receive dietary restriction. Tamoxifen causes varying degrees of decreased weight gains so our goal in dietary restriction of controls is to match the tamoxifen-treated animals for rate of weight gain.

The animals treated with Tamoxifen as a chemopreventative agent are now approximately one year post-irradiation. These results will be discussed in detail in the Final Report for the Chemoprevention Project, but

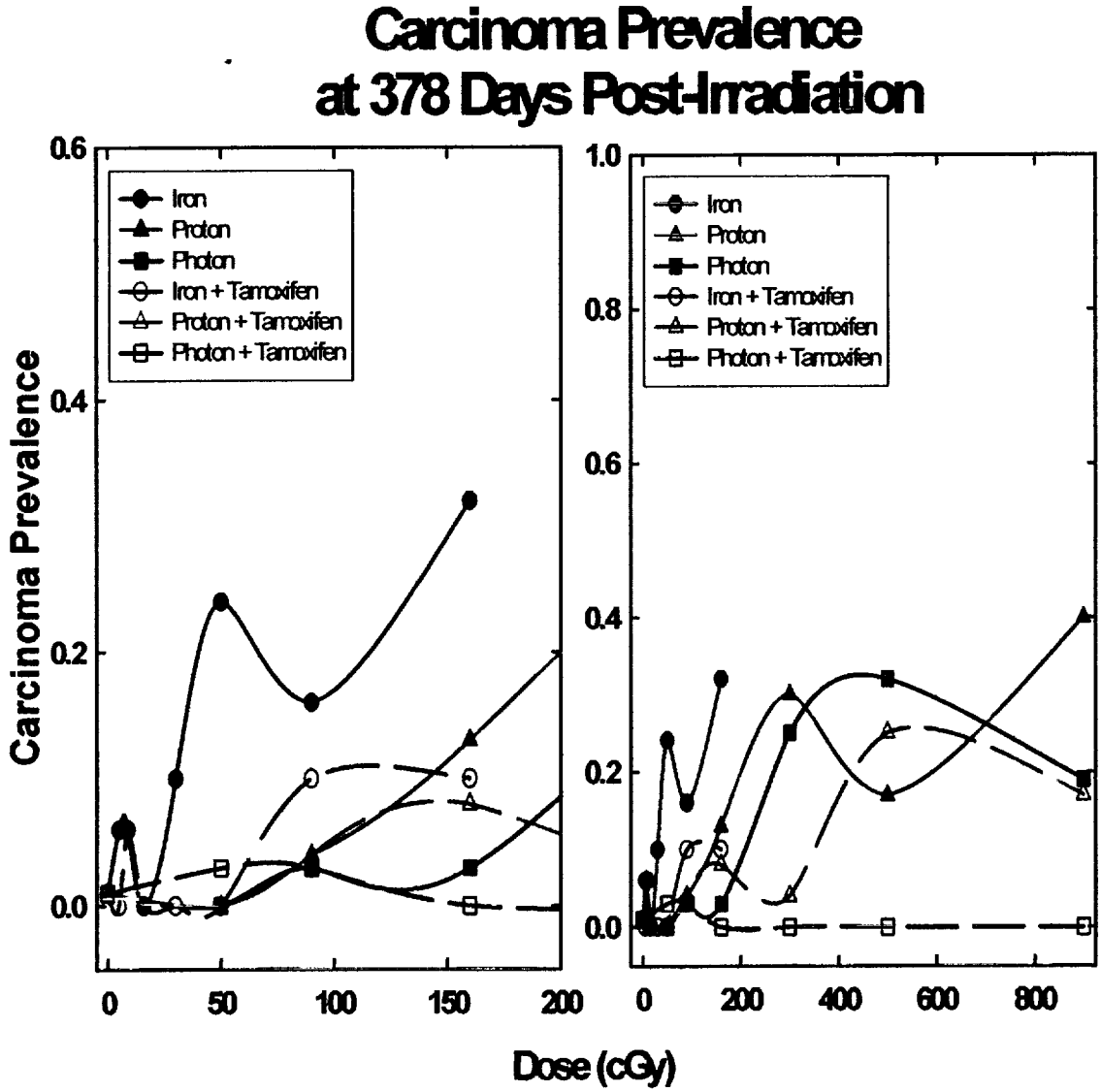


Fig. 10. Prevalence of cacinomas with and without subsequent Tamoxifen.

the care of these animals and the analysis of the data are a major commitment for this project. For that reason, a brief summary is presented here. Figure 10 shows the carcinoma prevalence at 378 days post-irradiation. It is clear that Tamoxifen reduces the occurrence of mammary carcinomas at higher radiation doses. The Tamoxifen is less effective on the iron-irradiated animals. Interestingly, proton irradiated animals do not show as dramatic a reduction as photons, perhaps as a result of LET or track structure differences of the protons or their secondary radiations.

4. *To evaluate response to rat mammary epithelial cells irradiated in vitro and in vivo and human epithelial cells irradiated in vitro with photons, protons, and iron ions and compare these responses with other cell types.* Completed (Cytogenetics Project).

This project has made several key findings that describe, in detail, chromosomal damage induced by three model space radiations: photons, energetic protons and energetic iron-ions (Fe-ions). The large number of data produced provide new insights into the relative potency of these radiations to induce different types of aberrations in multiple cell types: **human lymphocytes, human mammary cells, rat mammary cells (in vivo and in vitro) and multiple forms of human colorectal tumor cells** that have specific modulations of cancer-relevant genes. Further, we have investigated the induction of these radiations when used in fractionated or protracted time patterns in human lymphocytes, these data providing a paradigm for extrapolation of data from acute exposure to other exposure patterns. Further, the analysis of multiple aberrations per cell may be a "signature" for the dose and quality of radiation that induced these damages. We observe specific changes in induction of chromosome aberrations in cells that are deficient in expression of p53, p21 and 14-3-3-sigma, demonstrating that early changes in cells may render them more susceptible to further genetic damage induced by the three model space radiations. These data also provide new insights into the mechanisms of molecular biology by which cells process radiation damage. We have also measured clustering of multiple aberrations in individual cells so that Poisson analysis can be used to consider the relative influence of radiation quality on multiple events. Finally, we have suggested a new model for the dose response of cells to these radiations, focusing on induced cellular processing of radiation damage. Our model, that we term the subalpha- alpha-omega (SAO) model, provides a new structure for mathematical paradigms for testing whether chromosome aberrations can be used as a surrogate marker in estimating cancer risk. Our data on chromosome aberrations, when combined with the outcome of parallel studies in carcinogenesis in the Sprague-Dawley Rat, will provide a direct comparison between the rate of induction of chromosome aberrations in mammary epithelial cells with the rate of induction of mammary cancer in the same animal model over the same dose-ranges.

Our research activity is described in eight categories:

i) We have compared aberrations induced in different cell types by photons, protons and Fe-ions and defined five separate categories of aberrations based on cellular susceptibility.

When data for aberrations induced by the three radiations are compared across several cell systems, it is clear that analysis should be segregated into five types of aberrations: i) gaps and breaks in chromatids {G+B}, ii) acentric fragments {AcF}, iii) dicentrics and rings, {D+C}, iv) complex aberrations {CCA}, and v) premature centromere separation {PCS}. For each of these five types of aberrations, at least one cell type shows unusual susceptibility to one type of aberration but not others, demonstrating that the genotype can "uncouple" cellular processes that lead from initial radiation damage to manifestation of aberrations. For induction of CCA for instance, increased susceptibility is limited to a single cell line (SW1222) in which expression of p53, p21 and 14-3-3-sigma, as measured by Western analysis is not observed in unirradiated or irradiated cells. Similarly, increased levels of premature centromere separation is also observed only in a single cell line, a double knockout of p53 in a previously wt p53 cell. Thus our analyses will focus on induction of three types of aberrations: P+B, AcF and D+R.

ii) We have documented common dose-response patterns across all cell types.

While there is substantial variation between induced aberrations in different cell types and between Fe-ions and photons or protons, a common pattern can be observed for dose-response patterns for B+G, and a separate dose pattern for AcF and D+R. Background levels and relative numbers of induced lesions is cell type specific but our data shows a general pattern for all cells for all radiations with Fe-ions showing increased efficiency for AcF and for D+R.

ii a) We document a "plateau" type dose-response pattern for induction of G+B in all cell types for all three radiations.

For G+B there is a significant background level in all cells and small doses (0.01 Gy) of photons and Fe-ions induce significant increases. These increases reach a plateau of response at approximately 0.05 Gy and remain relatively constant over extended dose ranges. Three genetically-manipulated human colorectal tumor cell lines have increased background levels of B+G compared to their parent lines. Two of these cell lines (80S4 and 19S184) are double knockouts of CDKN1A (p21) in parent lines HCT116 and DLD-1 respectively and the third line (14-3-3-sigma -/-) is a double knockout of 14-3-3-sigma in HCT116. Interestingly, the line 379.2, a double knockout of TP53 (p53) in HCT116 cells and SW1222, a cell line that does not express p53, p21 and 14-3-3-sigma, do not show elevated background levels of B+G.

ii b) We have documented a "three component" dose-response pattern for AcF and D+R in all cell types for all three radiations.

For AcF, double chromatid fragments of equal length without a centromere, and for R+D there is a relatively similar general pattern, however differential quantitative susceptibility between cell lines and between radiations suggest to us that we should consider these two forms of aberrations differently. This common pattern is best described as a three component pattern, each response sequentially observed as dose is increased. In some 15 of our dose response patterns, there is an indication of additional structure at very low doses (below 0.05 Gy) however further statistical analysis must be performed before such structure can be confirmed in this low-signaloregion. The three-component pattern is clearly and most easily discernable after Fe-ion irradiation and for cell types that are more susceptible to the induction of these aberrations. The dose response pattern can be divided into three components for Fe-ions, each limited to specific dose ranges, and to three components for both photons and protons which is observed at higher doses, at least for transition to the second and third components. However the shape is similar: an initial rise, a subsequent decrease in rate and then a transition to a higher rate. For both AcF and R+D there is an initial increase from extremely low levels in most unirradiated cells to a change in the rate of induction at approximately 0.25 Gy for Fe-ions and approximately 0.5 for photons. We will refer to this range of doses as the subalpha response. This reduced rate dominates until approximately 1.0 Gy for Fe-ions and between 1.0 and 2.5 Gy for photons and protons. We term this range of response the alpha response. As our standard challenge doses for both photons and protons were given at 1.0 and 2.5 Gy, the exact definition of the dose at which the rate of induction acclerates is difficult to estimate for these radiations. This final rate of rapid increase for AcF and for D+R we term the omega phase. The omega phase varies considerably between cell types and for cells with certain genetic deficiencies.

iii) We have shown that the dose-response patterns for induction of chromosome aberrations may correlate with the induction of other cellular endpoints and with induction of cancer.

We have demonstrated that the three component (subalpha-alpha-omega) model that we observe for the induction of chromosome aberrations may also underlie the patterns observed for cellular mitotic death, for induction of mutation and in-vitro transformation and for cancer and have published these correlations (Williams et alia, 1998). Cell types vary in their susceptibility to the relative induction of aberrations over the three dose ranges. We propose that comparisons between cellular and molecular endpoints and between cellular and molecular events and the induction of cancer will be best performed using this model.

iv) We have documented the response of peripheral human lymphocytes to graded doses of photons, protons and Fe-ions.

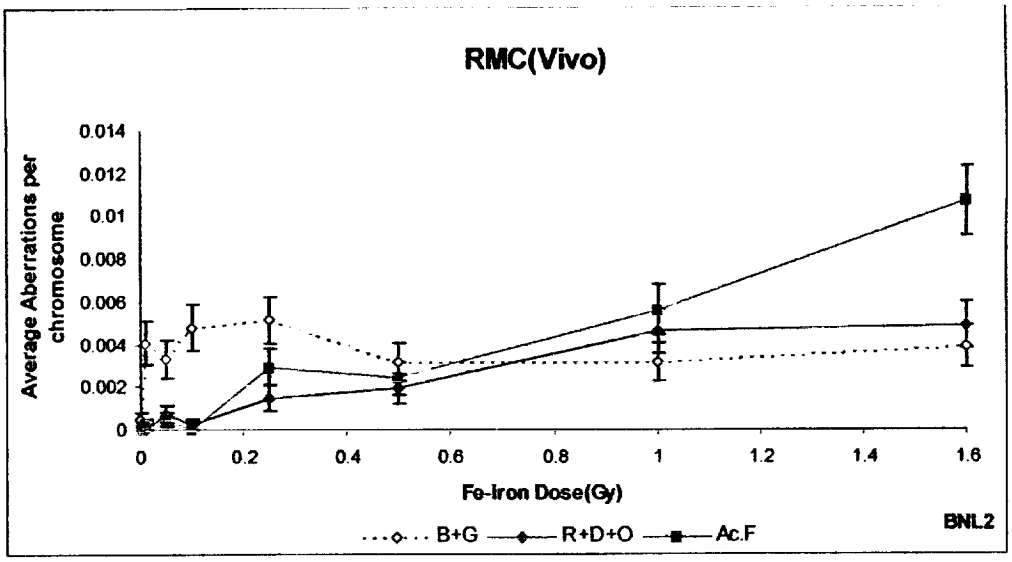
We have measured response of human peripheral lymphocytes derived from 17 "normal " donors when exposed to photons, protons and Fe-ions in quiescence and then assayed for aberrations in the first post-irradiation mitosis. We have measured the dose response for photons in 6 different donors, for protons in 4 different donors and for Fe-ions in four different donors. We have established background patterns and show that relative response to irradiation is not correlated with existing background patterns. These patterns show that the concept of RBE is not particularly useful in analysis. This data base will be important in the interpretation of chromosomal damage induced in lymphocytes of astronauts during space exploits.

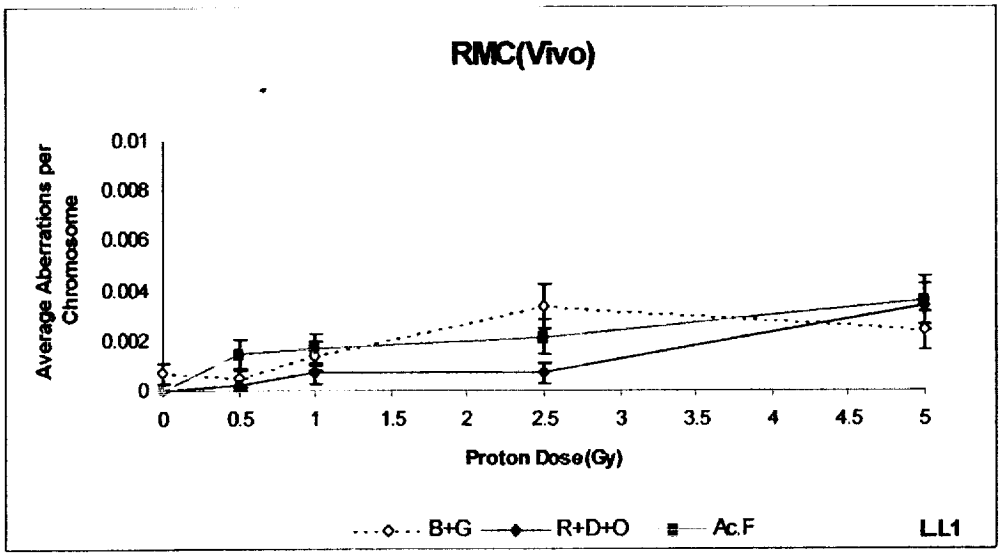
v) We have documented the response of three types of mammary epithelial cells to graded doses of photons, protons and Fe-ions.

We have measured the response of three types of mammary epithelial cells: the human established line MCF-10A and primary mammary epithelial cells of the Sprague-Dawley rat irradiated either in vivo and then transplanted for assay or irradiated in vitro after explant and assayed in vitro. These studies were done at the same time and at similar doses to animal carcinogenesis studies with the same animal. Our data show that cell irradiated in vivo are less susceptible to radiation-induced chromosomal damage but exhibit the same general pattern of response. Similar to 4 above, the concept of RBE is not effective as an analytical parameter.

vi) We have documented the response of human cells with genotypes relevant to multistage carcinogenesis to graded doses of photons, protons and Fe-ions.

We have measured the response of eight different human colorectal cell lines selected for their relevance to multistage carcinogenesis. Cell lines varied in their status in expressing p53 (wildtype, dominant negative or null), p21 (competent or deficient) and 14-3-3-sigma (competent or deficient) and for one cell line (SW1222) that does not express p53, p21 or 14-3-3-sigma. Cells with dominant negative p53 are less susceptible to the induction of AcF and D+R than cells with wildtype p53, suggesting a mechanism for the increased prevalence of mut p53 in human tumors. Cells with aberrant G2-M arrest (SW1222 and 14-3-3-sigma -/-) are hypersusceptible to some forms of chromosomal aberrations but not others. These data now offer a model system for more detailed studies on the molecular biology of DNA damage processing that leads to the different types of chromosomal aberrations. These data demonstrate that the genotype of particular cell types modulate the expression of all five types of chromosome aberrations that we have studied.





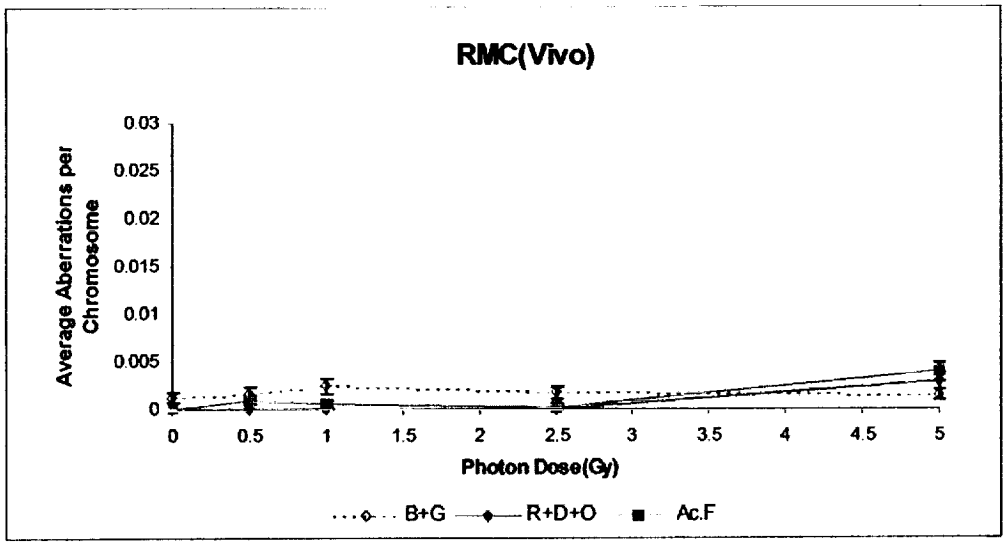


Fig. 11. Average aberrations per chromosome in rat mammary cells as a function of dose for iron, protons, and photons.

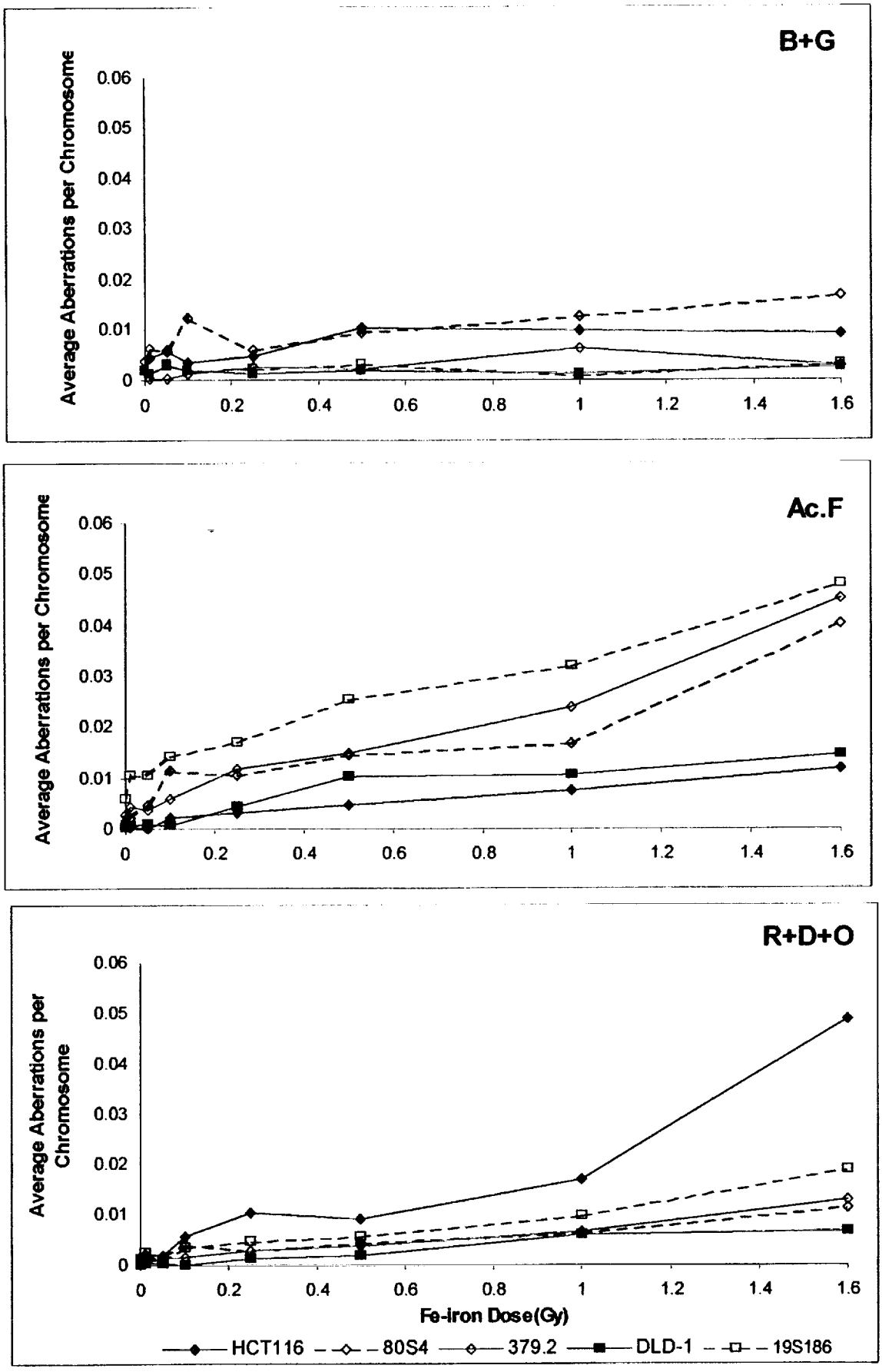


Fig. 12. The average aberrations per chromosome versus dose for the three types of aberrations.

vii) We have documented the response of human peripheral lymphocytes to fractionated and protracted irradiation compared to single acute exposure.

We have exposed quiescent human lymphocytes to either radiation delivered in multiple small fractions (10 fractions of 0.25 Gy or 20 fractions of 0.125 Gy each) or continuously for 10 hours at 0.25 Gy/hr or for 20 hours at 0.125 Gy/hr. Our data show that there is no sparing effect for either fractionation or protraction for the induction of B+G. However, for both AcF and D+R there is a substantial sparing for protracted irradiation but only a small, but significant, sparing effect for fractionation.

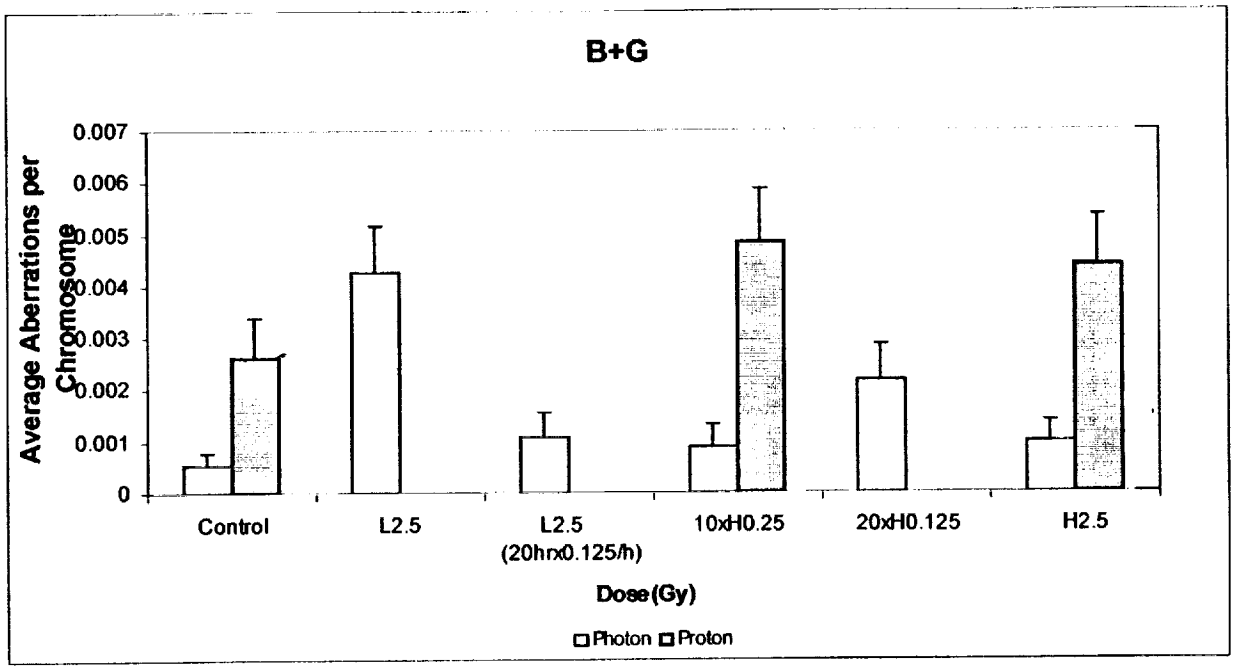


Fig. 13. Aberrations for different fractionations for photons and protons.

viii) We have documented the relative potency of photons, protons and to induce multiple aberrations in individual cells.

In all experiments we have evaluated each mitosis for the induction of all types of aberrations observable in solidly stained chromosomes. Thus we were able to measure the distribution of multiple aberrations in each cell as a function of dose and as a function of radiation type. Since the number and spatial distribution of ion clusters will vary for the three radiations (photons, protons and Fe-ions), we sought to compare the frequency distribution of multiple events as a function of dose for all three radiations. We observe that photons compared to protons and Fe-ions are most effective per unit dose for the induction of multiple aberrations (1, 2, 3, 4, 5....12 aberrations per cell). Fe-ions were significantly less effective in the induction of such multiple events than either photons or protons. These data will require further and extensive analysis for a better understanding of the spatial distribution of DNA "hits" for the three radiations. We suggest that the relative number of multiple aberrations in cells may be a "signature" for the type and dose of radiation to which the cells were exposed.

The implications of the findings listed above for future research are several and important:

a. **Our data offer immediate comparison with animal carcinogenesis data to determine whether chromosome aberrations are an appropriate biomarker for cancer risk.**
The data produced in this project was part of a team effort to measure effects of model space radiations in production of chromosome aberrations (this project) and in parallel experiments, in the induction of cancer. One of the major problems for assessing the carcinogenic effect of different types of space radiations on astronauts has been the absence of animal experiments of sufficient breadth and depth to establish a data base for comparison to the human data base on radiation carcinogenesis. The subsequent analysis of the data from this project and the parallel animal studies will partly rectify this deficiency. While data from a particular animal model is limited, the breadth of the present experiments provides a basis for more confidence in interpretation of the results and their relevance in human risk assessment.

b. **Our data offer new insights for mathematical models needed to analyze patterns induced cancer in animals and humans.**
Human carcinogenesis data is limited both in terms of the types of radiation that have been documented to cause cancer in humans; in the dose-time patterns to which humans have been exposed; and in the number of humans within the study cohorts; this last factor limiting precision of measurement. Experiments in animal carcinogenesis are also limited in practical terms in the number of animals that can be exposed which in turn limits the number of radiations and time/dose regimens that can be examined. Additionally the problem of whether a specific animal model is an appropriate for human response must be considered. Mathematical models have been used to extrapolate from animals to humans, from higher doses to lower doses and from animal response to human response. Such models are based on postulating the shape of the dose-response curve so that higher doses that usually can be determined more precisely, can be extrapolated to lower doses that are more relevant to human exposure. Thus the mathematical model must postulate that response at higher doses is predictive at lower doses. The most widespread model, the linear quadratic model, postulates that only two parameters-the coefficient of a linear component and the coefficient of a quadratic component- are needed to predict the shape of the dose-response curve at all doses. Our data strongly suggests that this model does not describe what we observe as a more complex response pattern. Our data show that while there are indeed general response patterns, a three-component model, the subalpha-alpha-omega, model not only describes the dose-response curves for the induction of AcF and D+R in all cell systems and for all radiations, but also is consistent with patterns of induction of cellular events and cancer in animals. We are still in the process of developing this model in terms of the set of parameters that are sufficient to define the dose-response pattern over all relevant doses. Whether this model proves to represent better the underlying mechanisms or not, it is clear that it will be useful in comparing different types of radiations and different biological systems.

c. **Our data establish an important data base for the response of different cell types to three model radiations.**
For instance, there is accumulating data on the prevalence of chromosome aberrations of different types in the peripheral lymphocytes of astronauts who have traveled in space. The data produced in this project will offer an excellent basis of comparison for these data. Similarly, the induction of chromosomal damage in other somatic target cells in human is extremely difficult to measure in situ or ex vivo. Thus the measurement of damage in an accessible cell such as the peripheral lymphocyte must be evaluated for response of other somatic cells that are known targets for radiation carcinogenesis, such as mammary or colorectal cells. The data base that we have accumulated directly compares the response of human lymphocytes to other somatic cells and compares human and rodent cell response.

d. **Our data demonstrate the need for extensive fractionation/protraction experiments these data show that fractionation and protraction, at least over the doses/dose-rates used, do not predict response to the space environment composed of different patterns of time/dose/LET.**
The space environment is extremely complex in terms not only of multiple types of particulate radiation that occur there but also in the patterns of dose and time that characterize radiation exposure. The concept of dose-rate as it is currently used through identifying a dose-rate-modifying-factor (DRMF) may be limited in describing the mixture of high LET which will have an extremely high instantaneous dose-rate, but a low average dose-rate. We approached this problem with limited experiments that compared the induction of multiple small fractions (0.125 and 0.25 Gy) to protracted irradiation at 0.125 and 0.25 Gy/hr. We observe the later to be mores sparing for the induction of chromosomal damage (at least AcF and D+R) than the former. While there is probably some size of very small fractions that when delivered repeatedly may be equally sparing as protracted radiation, it is clearly smaller than 0.125 Gy. The determination of the equivalence or lack of equivalence of small fractions and continuous irradiation needs to be determined to model molecular/cellular response to space radiation environments.

e. **Our data predict that the relative susceptibility to chromosomal damage and probably then to subsequent advancement toward overt carcinogenesis, will vary in cells that due to prior genetic changes are at different stages in multi-stage, multi-pathway carcinogenesis.**
Multiple genetic changes are needed to convert a normal epithelial cell to malignancy. Since many events, perhaps as many as 5 to 15 are needed in some types of cancer, the probability that the induction of multiple events in the same cell are independent is highly unlikely. Thus there is considerable thought that some pre-malignant changes predispose the changed cell to further changes. We have measured the response of human colorectal tumor cells to the induction of chromosomal damage by three model space radiation. These cells vary in their expression of at least three genes that are altered in tumor cells: TP53, CDNK1A and 14-3-3-sigma. We show that changes in these genes later the susceptibility to the rate of induction of different types of chromosomal damage. These data imply that space radiations may have differential effect on somatic cells that are already partially transformed. It seems reasonable to hypothesize that all astronauts have a substantial number of somatic cells that have undergone some premalignant changes. It therefore seems required to consider the relative impact of premalignant changes on the susceptibility of somatic cells to transformation to the overt cancer state by space radiations.

f. **Our studies establish a structure for studies on the molecular basis of chromosomal damage.**
Our studies described in 5 above also provide a useful first step in studying the molecular basis of cellular processing of radiation-induced damage. Since we have described the association of hypersusceptibility of cells with specific genetic deficiencies to specific types of chromosomal aberrations, extension of these studies to other types of cells with other changes in genes relevant to cancer is needed.

5. To irradiate mouse lungs in vivo with photons, protons, and initially, iron ions in order to evaluate tumor formation in mouse lungs with and without chemoprevention. **This proposal was not funded.**

6. To irradiate the same animal models with both energetic protons and iron, to evaluate the level of (predicted) synergistic, i.e., non-additive, effects of sequential or simultaneous irradiations, more closely approximating the space environment. **This strategic goal was reduced in scope prior to initiation of the project because of reduced funding and the complex logistics of irradiating the same animals at widely separated facilities. However, a preliminary experiment was successfully executed and the results are presented below (Core Project).**

Current radiation risk assessment practices generally assume that the total risk from a mixed radiation environment is simply the sum of the risks from each component. We are unaware of any in-vivo validation of this assumption as it relates to carcinogenesis and in-vitro experments are inconsistent with this assumption. We initiated a small pilot study whereby 11 rats were irradiated with 16cGy of iron and then two days later, 160cGy of protons. Our preliminary data, shown in Figure 14, demonstrate that there may be a greater response than what would be expected by the summation of risk from each dose component. The risk for each component was determined by linear regression of the dose response curves from the iron and proton irradiated animals. These data involve a small sample size and unresected tumors as opposed to classified tumors as in previous studies; however, it suggests that the study of a mixed radiation environment merits further evaluation.

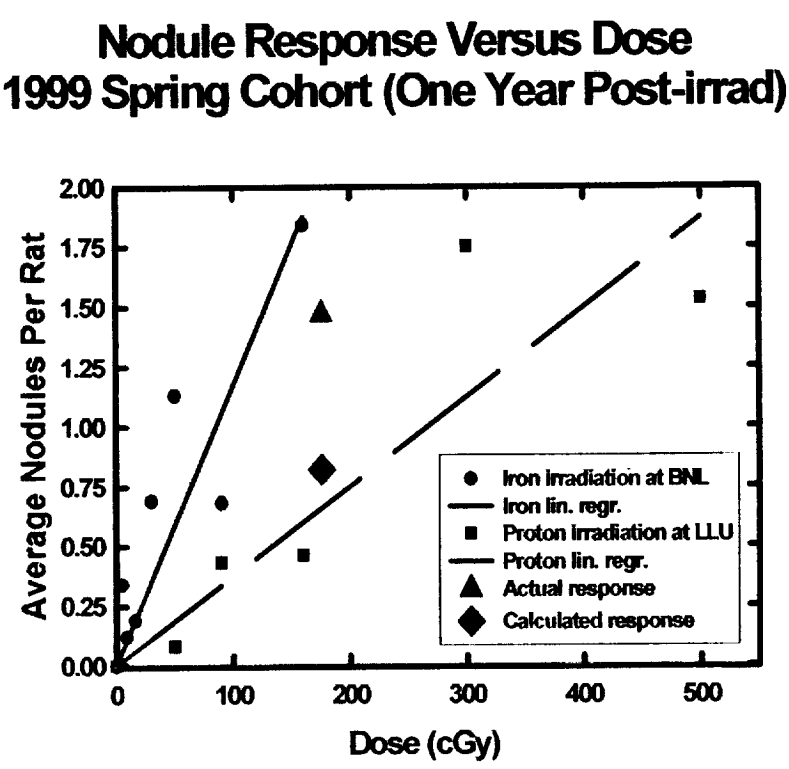


Fig. 14. A comparison of measured response for sequential irradiations with iron ions and protons with the expected additive.

7. To examine the changes in response with fractionation and changes in dose-rate. **This strategic goal also was eliminated prior to initiation of the project because of reduced funding. Again, a preliminary experiment was successfully executed and the results are presented subsequently (Core Project).**

A small pilot study of the effect of fractionation of the iron dose was initiated recently because doses in space will generally be protracted, and any assumption that fractionation does not alter carcinogenic response to HZE particles is subject to question. Five rats were irradiated with 10 fractions of 5cGy each, separated by approximately 20 minutes between fractions. We wish to compare their carcinogenic response with the acutely irradiated 50cGy animals. At the present time, too little time has passed to perform an analysis, but this group will be followed.

8. To model the biological results, so the data may be extrapolated to humans for the scenarios expected in space. **We are developing a new, non-linear model which we are successfully applying to our cytogenetic data and our animal model to calculate the risk of carcinoma. (Core Project).**

The construction of mathematical models that incorporate gene expression, protein interactions, and signal transduction with radiation response would provide a more mechanistic approach to model radiation damage and cell physiology. Recently, we have developed a mathematical model of G1 control that accounts for the behavior of the cyclins, cyclin dependent kinases (cdks), the pRb protein, and the transcription factor E2F/DP (Cucinotta *et al.*, 1997; Cucinotta and Dicello, 2000). This work is being done in strong collaboration with the scientists at the Johnson Space Center to be certain of relevancy and success in applying it directly to space issues when we are at that stage. Presently included in this model is the description of the two classes of cyclin kinase inhibitors exemplified by the p21 and p16 proteins, respectively. The new approach of Cucinotta and Dicello (2000) to consider gene expression in models of radiation sensitivity is described next. Necessarily, such an approach requires restricting the number of pathways being considered, multiple parameters describing the kinetics or simplifying assumptions to reduce the number of variables. In practice, it is a combination of these approaches with a strong dependency upon experimental biology to continuously validate the theory to the application. In other words, such models will have the greatest accuracy when the interpolation or extrapolation is not too far from the experimental benchmark. Therefore, it is essential that any such model rest firmly on a strong applicable experimental program. In response to previous suggestions by reviewers, we will provide some details of this subproject.

To have the most complete set of relevant data for the Spraque-Dawley rat tumor responses, we have been closely collaborating with Williams et al. (1996) to supplement that available in the literature. We first consider experiments that demonstrate the importance of cell cycle proteins in determining radiation responses and show how these complex interactions can be incorporated into mathematical of cell cycle progression (Cucinotta *et al.*, 1997) and radiation response. We describe mathematically the molecular kinetics of the proteins that regulate radiation responses including passage through the restriction point and the G1 arrest observed in wt p53 cells following energetic photon exposures. For space radiation exposures, radiation quality is a large concern, and the models required for risk assessment must consider particle track structure (Cucinotta *et al.*, 1996) in addition to genetic expression. Current knowledge of cell cycle progression and arrest in mammalian cells includes the description of the phosphorylation events of several protein complexes made-up of cyclins and cyclin dependent kinases (cdk) and regulation of tumor suppressor proteins. Phosphorylation delays occur during repair of damage from radiation. We have constructed our mathematical model starting with cell cycle progression in terms of the kinetics of protein complex formation and regulation. A more detailed description is provided in the Final Report for the Core Project.

In Figure 15, the cell-cycle control proteins under consideration are depicted. G1 progression (Lukas *et al.*, 1994) is controlled by cdk4 and cdk6 and the D cyclins (D1, D2, and D3). The later G1/S checkpoint is controlled by cyclin E and cdk2 (Ohtani *et al.*, 1995, Ohtusubo *et al.*, 1995, and Koff *et al.*, 1992). S phase progression subsequently requires cyclin A binding to cdk2 and cdc2. The G2/M transition is controlled by cdc2 and members of the cyclin B family. The cyclin/cyclin-dependent kinases phosphorylate members of the Rb family (pRb, p107, and p130). After these molecules are phosphorylated, sequestered E2F-family proteins are released and initiate DNA transcription for the successful completion of the cell cycle (Welsh and Wang, 1995). Both cyclin E and cyclin A are induced strongly by E2F1, and the identity of other E2F responsive genes is currently under study (DeGregori *et al.*, 1995). The cdk inhibitors respond to external signals to control cell cycle progression by binding directly to cdks or blocking the phosphorylation of cyclin/cdk complexes (Tam *et al.*, 1994, Lukas *et al.*, 1995, and Dulic *et al.*, 1994).

The mathematical description (Cucinotta *et al.*, 1997) of G1/S control is given in Table 2. The time dependent concentrations of the cyclin, cdks, and cyclin/cdk complex's are described through their synthesis, degradation, and binding interactions.

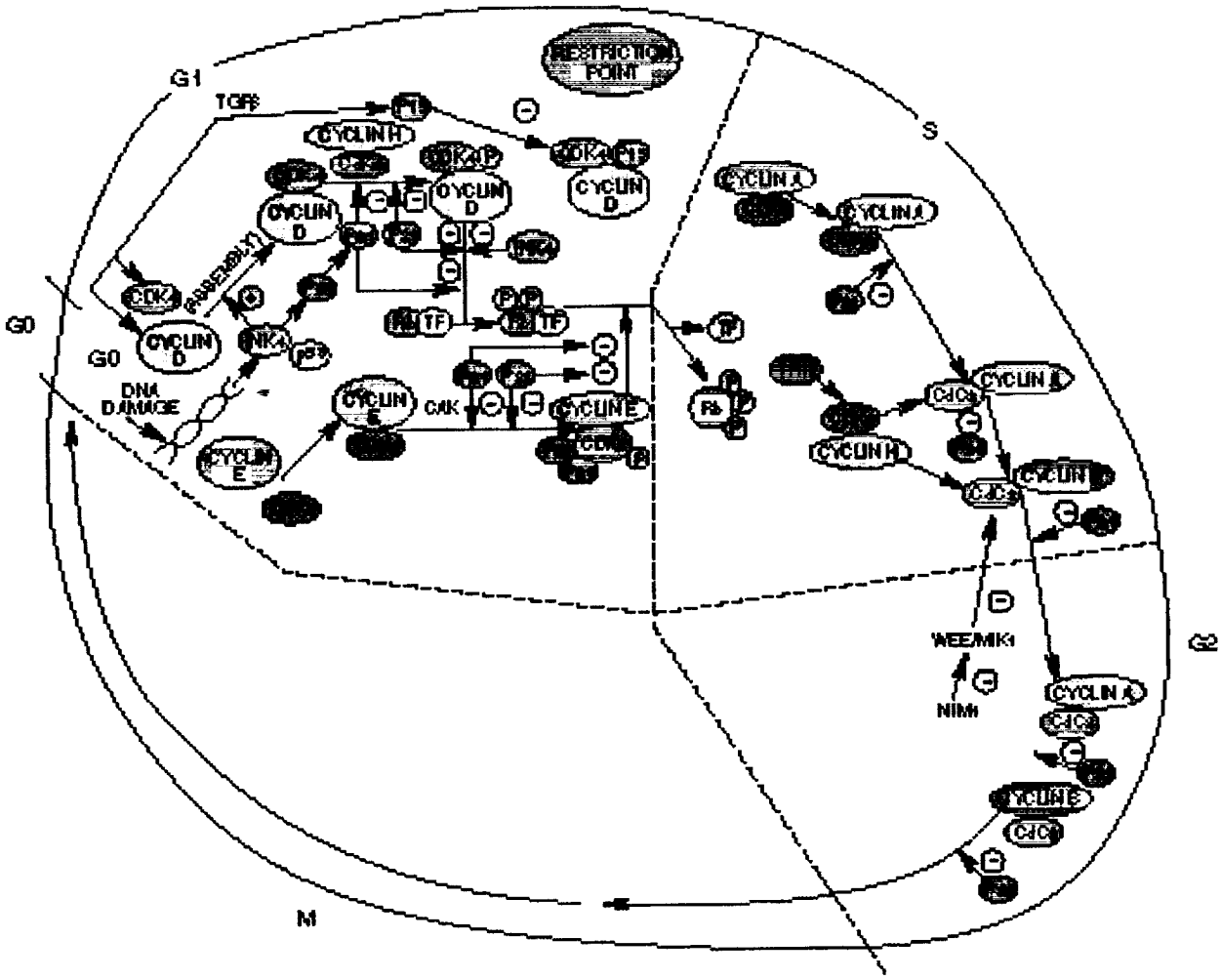


Fig. 15. Diagram illustrating functional roles of cell-cycle regulation proteins (Adapted from Hirama and Koeffler, 1995).

Table 2. *Molecular Kinetics Equations for Cell Cycle Progression and Regulation. The k_{ij} are rate constants for binding of molecules i and j, v_S and $v_D(v^P_D)$ are synthesis and degradation rates, respectively. The k^P (r^P) and k^Q (r^Q) are rates of phosphorylation or de-phosphorylation, respectively. The $k_{F\,i+1}$ is rate of re-formation of a pRb/E2F complex denoted [pRb-E2F]. The cyclin associated kinase complexes are labelled with superscript I = 0,1,2,P for activity of phosphorylation sites. Concentrations of molecules with P superscripts are the activated form and those with superscript 0 are the inactive forms. pRb is assumed to be de-phosphorylated by a G2/M cyclin/cdk complex.*

$$d[Cyc_i]/dt = v_{Si}[TF_i] - v_{Di}[Cyc_i] - \Sigma_j\, k_{ij}[Cyc_i][cdk_j]$$

$$d[Cyc^P_i]/dt = \Sigma_j\, v_{Dij}[M^P_{ij}] - v^P_{Di}[Cyc^P_i]$$

$$d[cdk_j]/dt = -\Sigma_i\, k_{ij}[Cyc_i][cdk_j] + \Sigma_i\, v_{Dij}[M^P_{ij}] - \Sigma_\alpha\, k_{j\alpha}[cdk_j][Ink_\alpha] + \Sigma_\alpha\, v_{D\alpha}[Ink_\alpha\text{-}cdk_j]$$

$$d[M^I_{ij}]/dt = k_{ij}[Cyc_i][cdk_j]\delta_{I0} - v_{Dij}[M^I_{ij}] - \Sigma_J\, \varepsilon_J\, k^{P,Q}_{ij}[M^J_{ij}] + \Sigma_J\, \varepsilon_J\, k^{Q,P}_{ij}[M^J_{ij}]$$

$$- \Sigma_\beta\, k_{ij\beta}[KI_\beta][M^I_{ij}]\delta_{I0} + \Sigma_\beta\, v_{D\beta}[KI_\beta\text{-}M^0_{ij}]\,\delta_{I0}$$

$$d[pRb^P]/dt = \Sigma_j\, r^P_{ij}[M^P_{ij}][pRb\text{-}E2F_{i+1}]/(K+[pRb\text{-}E2F_{i+1}]) - r^Q_{G2/M}[M^P_{G2/M}][pRb^P]$$

$$d[E2F_{i+1}]/dt = \Sigma_j\, r^P_{ij}[M^P_{ij}][pRb\text{-}E2F_{i+1}]/(K+[pRb\text{-}E2F_{i+1}]) - k_{F\,i+1}[E2F_{i+1}][pRb^0]$$

$$d[Ink_\alpha]/dt = v_{S\alpha}[TF_\alpha] - v_{D\alpha}[Ink_\alpha] - \Sigma_j\, k_{j\alpha}[cdk_j][Ink_\alpha]$$

$$d[Ink_\alpha\text{-}cdk_j]/dt = k_{j\alpha}[cdk_j][Ink_\alpha] - v_{D\alpha}[Ink_\alpha\text{-}cdk_j]$$

$$d[KI_\beta]/dt = v_{S\beta}[TF_\beta] - v_{D\beta}[KI_\beta] - \Sigma_{ij}\, k_{ij\beta}[KI_\beta][M^0_{ij}]$$

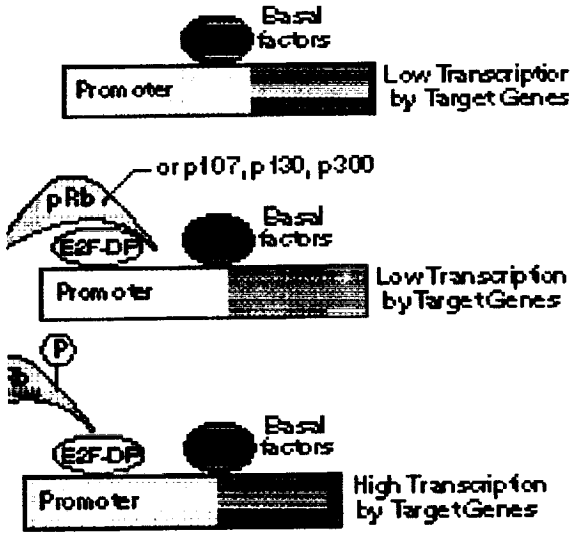


Fig. 16a. Diagram illustrating role of pRb and its phosphorylation and expression of transcription factors E2F/DP.

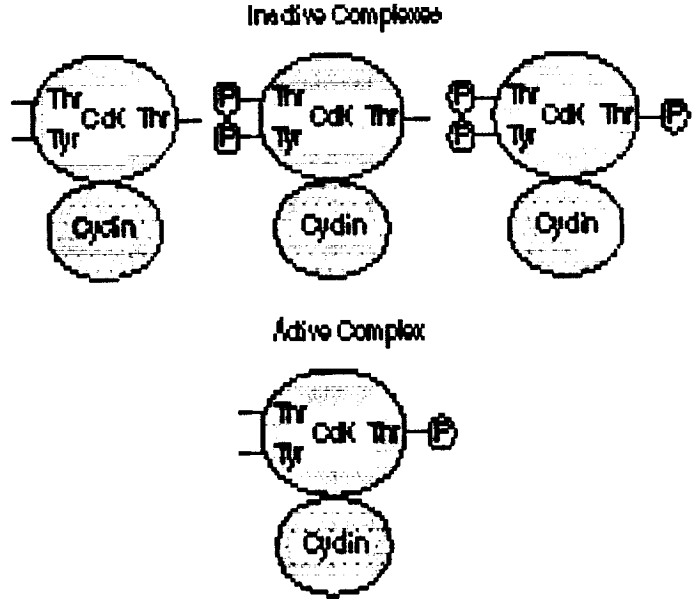


Fig. 16b. Diagram illustrating positive and negative controls on kinase activity of cyclin/cdk complexes.





The bound state of the pocket protein with the E2F/DP hetero-dimers acts as a substrate of cyclin-dependent kinases. In our model, we account for the release of the E2F's from pRb by cyclin kinases leading to transcription of E2F responsive genes as depicted in Figure 16a. To model this behavior mathematically requires an undamped oscillatory solution to the non-linear rate equation's which are known as limit cycle solutions. Figure 16b depicts the regulation of the cyclin/cdk complex. The phospatase molecules of the kinases provide both negative and positive control on the kinases. We follow the G2/M control model of Novak and Tyson (1993) and use rate constants for regulation of the negative residue that have a quadratic dependence on the kinase and assume that

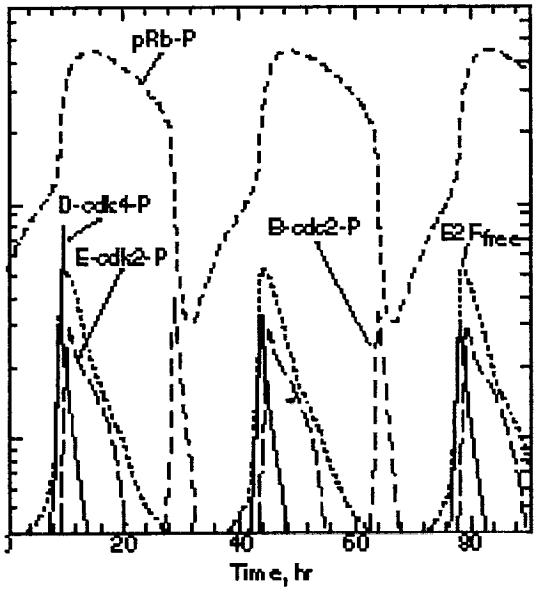


Fig. 17a. Calculations of cyclin E kinase in normal and Rb- cell lines showing proliferation (shortened G1 times).

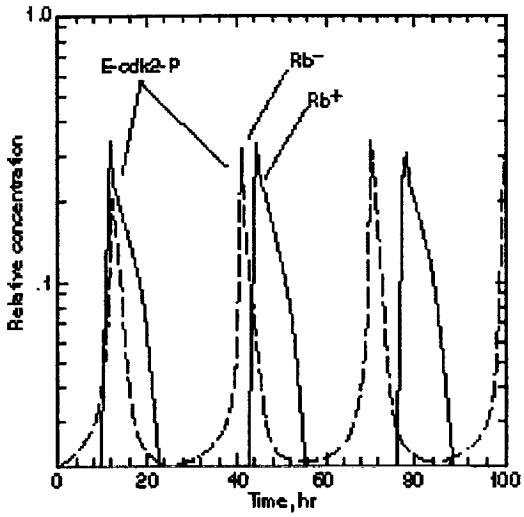


Fig.17b. Calculated percent expression of the cyclins D, E, and B as a function of time over several cell cycles showing limit cycle behavior found in the model.

the activity of the positive residue is in equilibrium with the de-phospatase. The degradation by ubiquitin-mediated proteolysis of the cyclins with the sequence homology known as the cyclin destruction box are assumed to have a rate-constant with a quadratic dependence on the active kinase. In Figure 16a we show the solution of the model for the time-rate of change of the D and E cyclins and the phosphorylation of pRb. In Figure 16b we show the increase in proliferation that occurs in the model in Rb deficient cells because of a decrease in G1 duration when E2F's are not regulated by pRb.

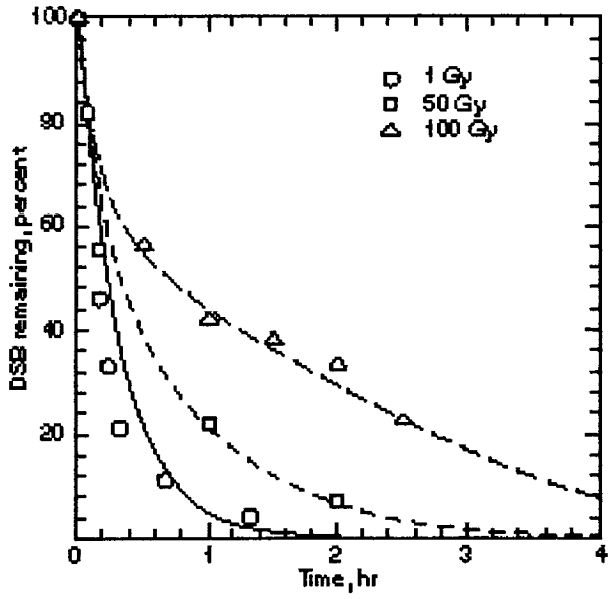


Fig. 18. Calculations of DSB repair versus time after exposure. Also shown are DSB repair data of McWilliams et al., 1983, and G. P. Van Der Schans et al., 1983.

P53 Regulation After DNA Damage

The tumor suppressor protein p53 accumulates after damage to DNA such as strand breaks caused by radiation exposure, by chemical agents that produce strand breaks, and following base excision repair which also produces strand break formation by a post-transcriptional mechanisms. (Nelson and Kastan, 1994). To consider p53 regulation in the model we consider the coupling of p53 directly to an enzyme complex for DSB repair. Single strand breaks (SSB) will

also regulate p53; however, since many SSB's are rejoined directly by ligases or repaired rapidly, we will model the longer lived DSB repair complex in our calculations. The half-life of p53 is 25 minutes. Coupling of the molecule to DNA damage will increase this signal by several hours depending on the level of damage and other modifying events. These include phosphorylation of p53 by molecules that regulate its DNA binding activity and proteolysis. Molecules responsible for phosphorylation of p53 include raf, DNA-PK, PKC, and cdk-cyclin complex's (Steegenga *et al.*, 1996). The latter may be involved in a feedback control mechanism, since the inhibition in activation of several cyclin associated cdk complexes is through transcription of p21 by p53.

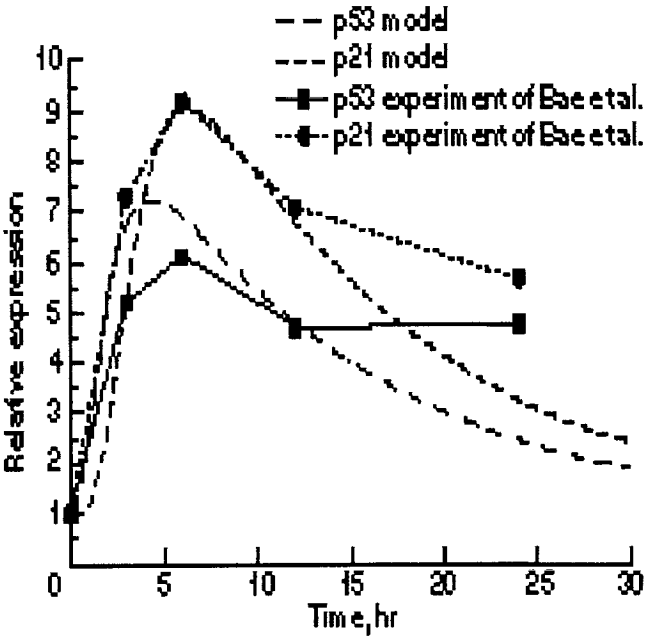


Fig. 19. Model calculations of p53 and p21 expression versus time after 6.3 Gy acute exposure as fit to data of Bae et al., 1995.

Following irradiation, the number of DSB's for energetic photon exposure occurs at the rate of 20–50 DSB/Gy. A heuristic model for the post-transcription modification of p53 is made by coupling p53 to the DNA repair complex. The repair complex is formed by radiation induced DSB's as

$$\text{radiation} + [\text{DNA}] \rightarrow [\text{DSB}] \qquad (1)$$

$$[\text{DSB}] + [E_{rep}] \rightarrow [C_{rep}] \rightarrow [\text{DNA}] + [E_{rep}] \qquad (2)$$

The regulation of p53 through the DSB repair complex, $[C_{rep}]$, is described as

$$d[p53]/dt = v_S - v_D[p53] - k_{p53}[p53][C_{rep}] - \{\text{other interactions}\} \qquad (3)$$

An effective half-life of p53 can then be defined as

$$1/\tau_{p53} = v_D + k_{p53}[C_{rep}] \qquad (4)$$

which leads to a dose dependent increase of the half-life over the base line value. We are ignoring the kinetic step of tetramerization of p53 since it occurs rapidly in several minutes or less. The repair of DSBs proceeds with first-order kinetics at low doses and a mixture of first and zeroth-order kinetics at higher doses as the number of repair enzymes becomes comparable to the number of break's (Lett 1994). In Figure 17 we show the comparison of the model to DSB repair data (McWilliams *et al.*, 1983 and G. P. Van Der Schans *et al.*, 1983). The enzyme repair model gives a good representation of the data for the doses shown. Several future considerations of the model include the possibility that the expression of the repair enzymes is regulated by the exposure such that exposures above about 0.5 Gy are not indicative of the repair system at lower doses or dose-rates studied here. Although p53 regulation has been shown to involve damage recognition of strand breaks (Lu and Lane, 1993, Nelson and Kastan, 1994, and Reed *et al.*, 1995), its regulation needs to studied as a function of cell cycle position and radiation type.

G1 Arrest Through the Kinase Inhibitor p21

The kinase inhibitor p21 is normally found in quaternary complexes with the cyclins, cdk's, and proliferating cell nuclear antigen (PCNA) (Namba *et al.*, 1995 and Li *et al.*, 1994). Increases in p21 concentration cause G1 arrest

(Dulic *et al.*, 1994) through inhibiting G1 cyclin associated kinase activity. In our model we examine p53 dependent p21 upregulation after DNA damage as described by

$$d\,[mRNA]/dt = r_T + r_{p53}\,[p53] - r_D\,[mRNA] \qquad (5)$$

where r_T and r_D are the basal rates of transcription and degradation, respectively, and r_{p53} is the rate constant for coupling of the transcription factor p53 to the p21 promoter. The time rate of change of p21 is given by

$$d\,[p21]/dt = v_S\,[mRNA] - v_D\,[p21] - k_I\,[p21]\,[M^0_D] - k_I\,[p21]\,[M^0_E] \qquad (6)$$

where v_S and v_D are the synthesis and degradation rates of p21, respectively. The last two terms in Eq. (6) are the coupling of p21 to the G1 cyclin-cdk complexes.

For the background levels of p21 we use its known half-life of 30 minutes and assume that there is normally about one molecule of p21 in each of the G1 cyclin complexes. Fits of the model to the data of Bae *et al.* (1995) for the time course of p21 and p53 expression after the exposure for normal lymphoblast cells are shown in Figure 18. Modifications of the present model will be required to describe the modulation of these proteins for time courses greater than 10 hrs after DNA damage, including the role of phosphorylation or other conformation changes of p53 throughout the cell cycle that are known to affect its DNA binding and transcription activity. Also, genomic instability after radiation exposure would likely modify p53 levels because of the observed high sensitivity of p53 to DNA damage (Nelson and Kastan, 1994). In Figure 20 we show the number of DSB remaining and the relative expression of p53 and p21 versus time for low dose-rate exposures. These low dose-rate results predict that there is no threshold for p53 induction in agreement with the review of Lane (1996) for low dose-rate acute exposures.

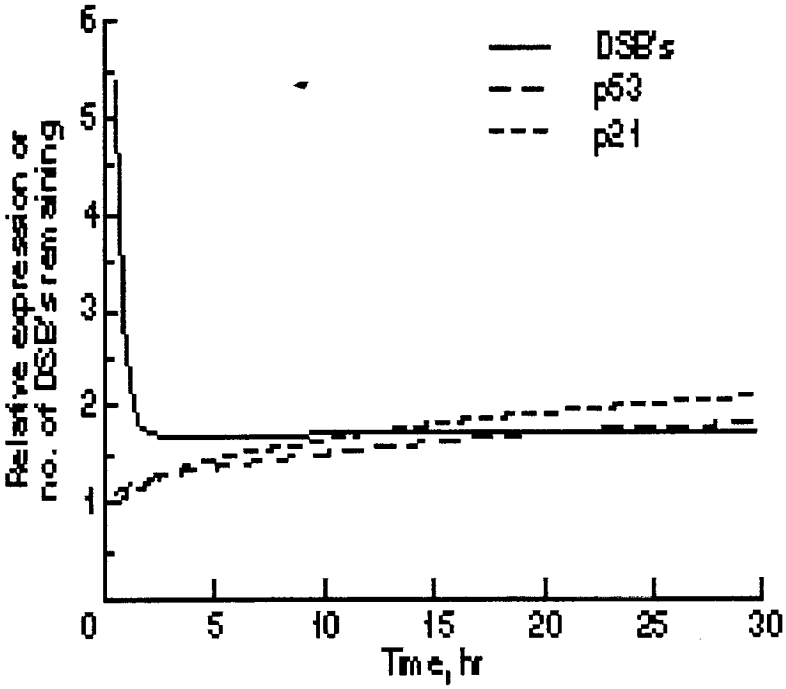


Fig. 20. Predictions of model for number of DSBs remaining and relative expression of p53 andp21 for 0.1 Gy/hr gamma irradiation.

In Figure 21 we show comparisons of the model for acute exposures of 6.3 Gy in early G1 and at G1 + 6hrs. The results in Figure 8a display a large G1 arrest in agreement with experiment (Dulic *et al.*, 1994). The results of the model shown in Figure 21a are in general agreement with the Western blot analysis of Dulic *et al.* (1994). The results of Figure 21b show only minimal G1 arrest for the same exposure level. Here, the non-linear expression of kinase activity prevents the inhibitor in causing arrest. These results suggest that for asynchronous populations, G1 arrest is dependent on timing of signaling pathways and non-linear accumulation of cyclin associated kinases.

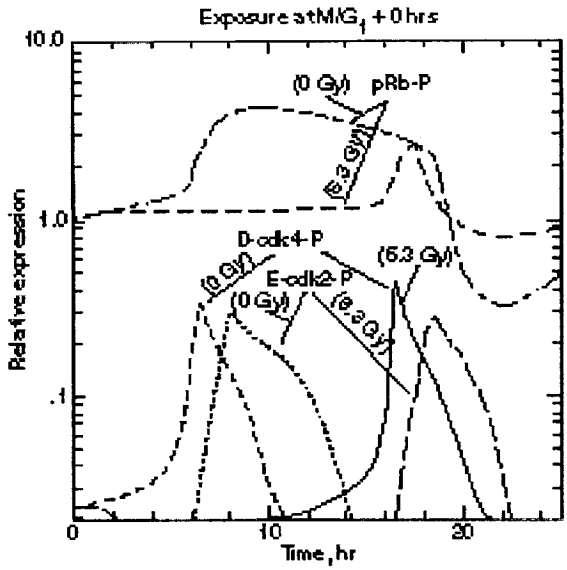


Fig. 21a. Calculations of relative expression of several proteins after acute exposure of 6.3 Gy at M/G1 border.

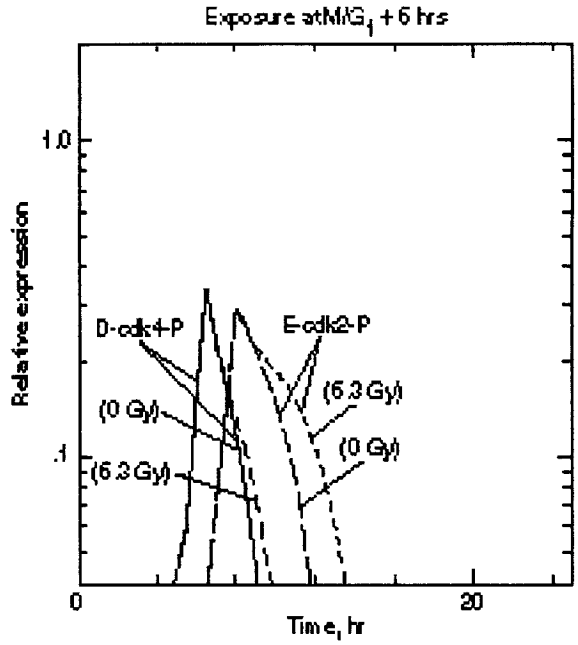


Fig. 21b. Same as Fig. 8a except it is exposed at M/G1 border plus 6 hrs.

9. To establish risks relative to low-LET radiations. **We have been careful that all of our experiments have had both unirradiated shams and photon-irradiated animals(Core Project).**

All experiments included shams and photon-irradiated animals for RBE analyses and for comparing with previous data. We used cesium-137 gamma rays at Johns Hopkins and BNL. We used cobalt -60 gammas at LLUMC. In each case the choice was determined by the only available sources. However, the evaluate the effects of transportation and different energy gammas, we irradiated half of our control animals at Johns Hopkins for each series.

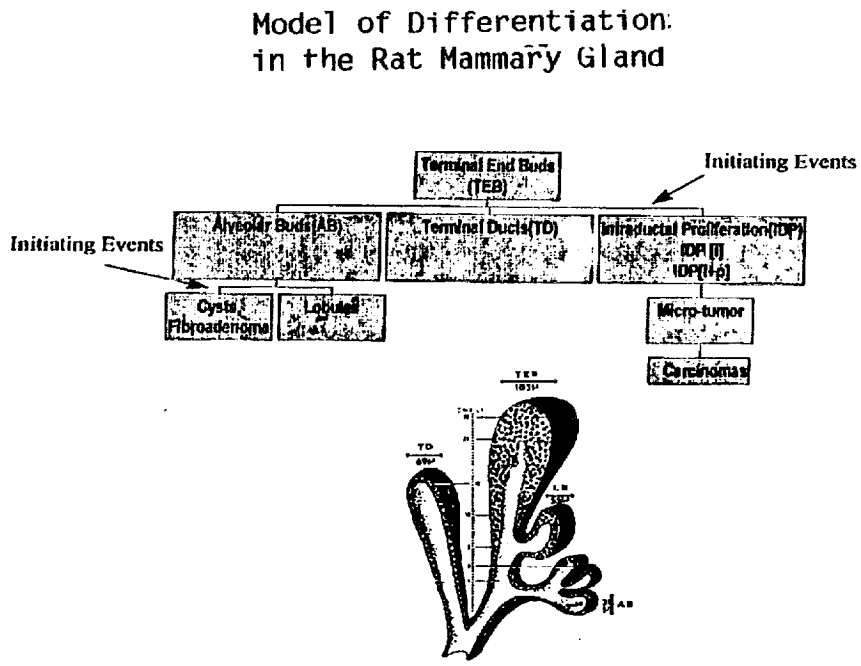


Fig. 22: A schematic of the pathways underlying the mathematical model used to simulate mammary-tumor induction in the Spraque-Dawley rat, according to the paradigm of Russo et al., (1983).

We described earlier a mathematical model of the interactions of cell cycle control proteins including cyclins, cdk's, pRb, E2F's, cdk inhibitors, and growth factors. The model has the potential for describing complex multstage processes such as cancer pathways. We have already applied it to calculate mechanistic-based cancer-risks for different tumors for the different radiation types and exposures of interest. As outlined in Figure 22, Russo and Russo

(1987, 1996) and Russo et al. (1983) characterized differentiation in the rat mammary gland and its response to a chemical carcinogen, DMBA. We use our mathematical model of molecular events in cell cycle progression to develop a quantitative model that characterizes differentiated structures of the mammary gland and initiation in control populations. We apply this model to describe the growth kinetics of undifferentiated structures in the rat mammary gland. The progression stage is believed to result from a differentiation of terminal end buds (TEBs) into alveolar buds (ABs) and lobules. Alternatively, the terminal end buds can regress to terminal ducts (TDs) as seen with aging. The pathways leading to both benign and malignant tumors are illustrated schematically in Figure 22. Young virgin rats are known to have the highest cancer risk. This increased sensitivity is attributed to their larger number of TEB's which possess a characteristically short G1 phase, and the higher sensitivity of S phase cells. The model is based on rate limiting transitions manifested in the activation of cyclin-cdk complexes through covalent modification and controls on cyclin transcription. We simulate the uncontrolled expansion of undifferentiated structures in the mammary gland using known mutational events that alter cell cycle controls, including pRb, p16 mutation and cyclin D over-expression.

The model assumes that entry into the cell cycle is stimulated by mitogen activation of cyclin D, and down-stream activation of cyclins E, A, and B with transcription control by E2F's sequestered by pRb. Application of this model to observations of different cell lineages in the rat mammary gland was achieved by modifying control of cyclin D expression in order to modify the length of G1 phase.

The same non-linear differential equations describing this model are shown in Table 3 and are applied to the mammary gland using the rate constants and the cell-cycle parameters, presented in Tables 4 and 5, which were determined from data in the literature.

Table 3. *Model Rate Parameters and Experimental Values. Model rate parameters are given for synthesis and degradation of cyclins and p16 and exp. degr. constants based on reported ½ lives. Degradation rates for cyclins are for activated cyclin/cdk complex.* $r^P = 5\ hr^{-1}$ *and* $r^Q = 0.1\ hr^{-1}$ *for cyclin/cdk phosphorylation of [pRb-E2F]. Other rate constants;* $k^P_2 = 180\ hr^{-1}$ *and* $k_F = 0.6\ hr^{-1}$ *for each i,* $k_{p16,cdk4} = 12\ hr^{-1}$.

Protein	[TFi]	$_{vs,}$ model	$_{vd,}$ model	$_{vd,}$ expt.	k^P_o
[Cyclin D]					
-TEB	mitogens	0.5,hr^{-1}	½, hr^{-1}	½, hr^{-1}	0.03, hr^{-1}
-TD	mitogens	0.15	½	½	0.03
-AB	mitogens	0.1	½	½	0.03
[Cyclin E]	[E2Fe]	1.08	1/3	-	0.015
[Cyclin A}	[E2Fa]	0.38	1/4	1/4	0.015
[Cyclin B]	[E2Fb]	0.53	0.075	0.067	0.09
[p16]	[TF_{16}]	1.1	0.125	<1/3	-

Table 4. *Cell-Cycle Parameters from Russo and Russo (1987)*

Group	Structure	Gl, hr.	S, hr.	G2, hr	M, hr.	T_{cycle},hr	Growth Fraction
Young Virgin	TEB	.72+/.42	7.2+/.80	1.08+/.11	1.2+/.2	9.93+/.31	0.55
	TD	7.5+/.12	7.5+/.83	1.00+/.01	1.4+/.4	17.33+/.42	0.39
	AB	17.3+/1.1	8.7+/.61	1.00+/-.01	1.2+/.2	28.2+/1.98	0.13
Old Virgin	TEB	5.2+/.99	7.6+/.40	4.40+/-.40	1.6+/.2	18.8+/0.99	0.077
	TD	7.1+/.08	7.6+/.40	4.40+/-.40	1.4+/.2	20.57+/1.8	0.020
	AB	17.3+/1.4	8.2+/.72	3.60+/-.40	1.7+/.1	30.75+/-2.7	0.008

We have applied the model to a situation of importance in designing our experiments, but for which there are insufficient data available. Because of the logistics of moving the animals and personnel to and from the various facilities used for the irradiations and the impossibility of doing all of the irradiations simultaneously, it is essential to understand how significant are the variations in response with the age of the animals at the time of irradiations. Figure 23 illustrates that the model is capable of adequately describing the observed variations in the number per unit volume of the critical structures with time as measured by Russo and Russo (1987, 1996). Then the results of the model for the variation in the development of mammary carcinomas as a function of time and dose for photon irradations are compared with the data of Johnson et al., (1989) in Figure 24. The incidence rate of the Sprague-Dawleys as a function of age was calculated and is presented in Figure 25. There are no data in the literature, so these were truly predictive results, results which we needed to determine the number of animals per dose point, when to irradiate, and how much time variation we could tolerate, knowing the large accelerator facilities are unreliable for meeting schedules set months in advance so we could order animals.

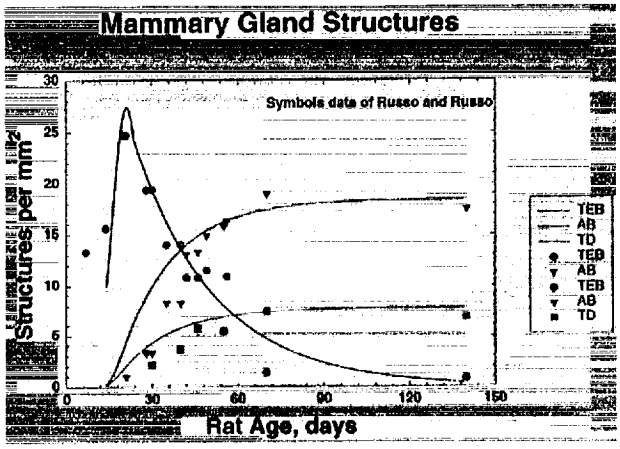


Fig. 23: A comparison of the theoretical model (solid lines) with the experimental results (symbols) for the progression and regression of differentiation of the rat mammary gland for the terminal end buds (TEBs), the alveolar buds (ABs), and the terminal ducts (TDs).

Finally, preliminary results of a maximum likelihood analysis which we (Lief Peterson, Baylor, Cucinotta, and Dicello) are in the process of developing are presented in Figs. 26a and 26b. This analysis is based upon a proportional-hazards model for relative risks of mammary tumors based and accounts for all radiation types and dose levels. Survival experience among cesium dose groups form the baseline for the survival experience.

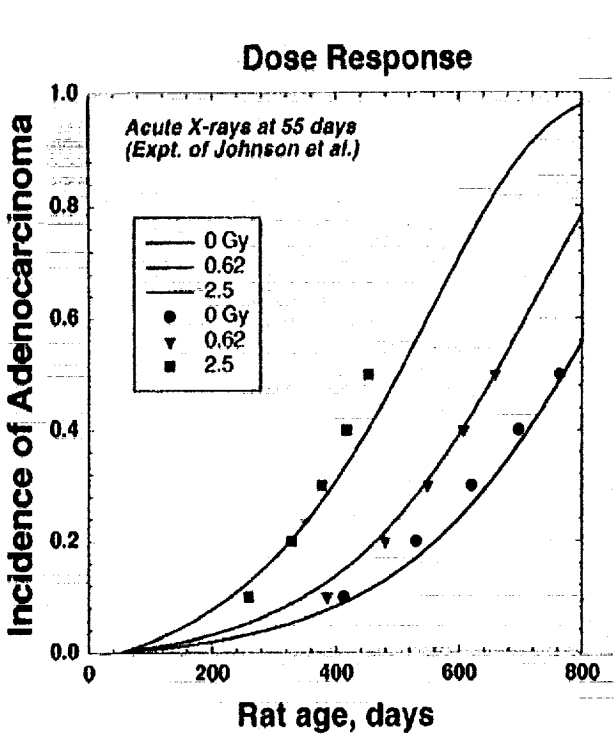


Fig. 24: A comparison of the incidence of adenocarcinomas in the rat mammary gland for the theoretical model (solid line) and the experimental results (symbols) of Johnson et al. 1989).

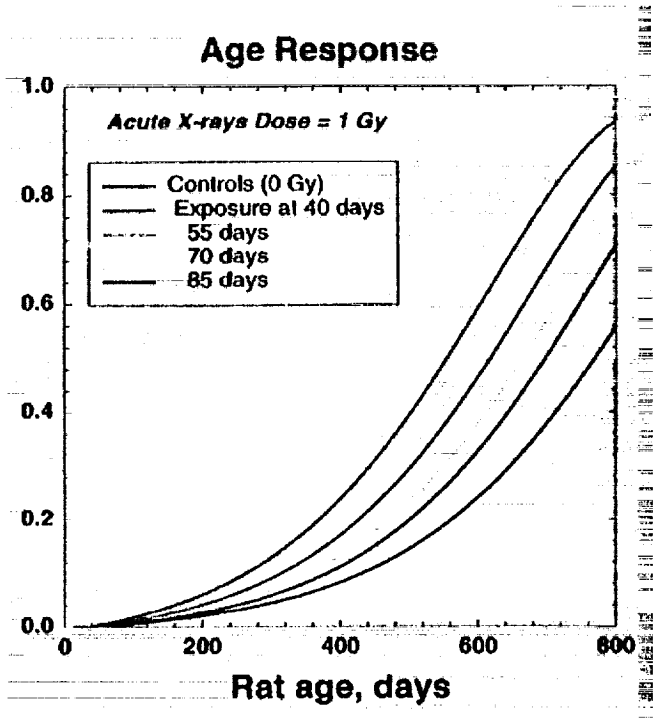


Fig. 25: An estimation of the change in the incidence rate as a function of age of the rats at the time of exposure of 1 Gy of photons.

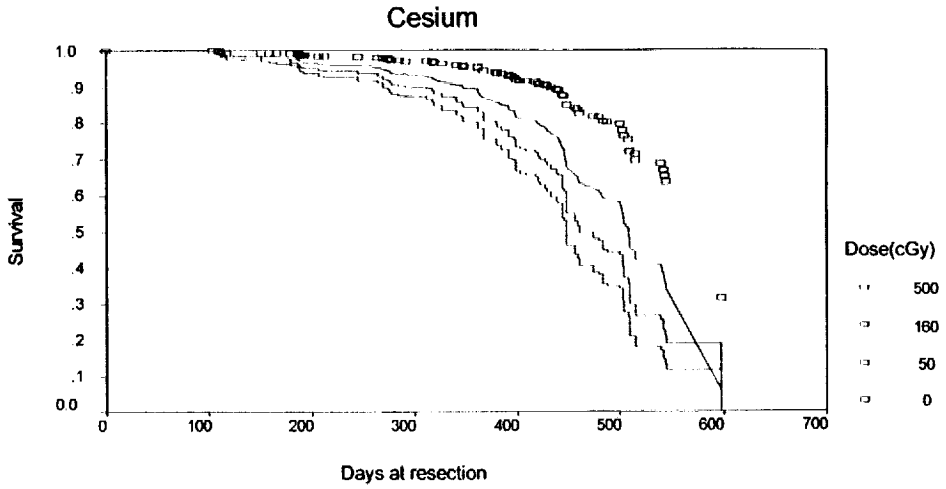


Fig. 26a. Preliminary results of a maximum likelihood analysis for relative risk for tumor-free survival as a function of time after irradiations with cesium-137 gamma rays. The cesium groups formed the baseline.

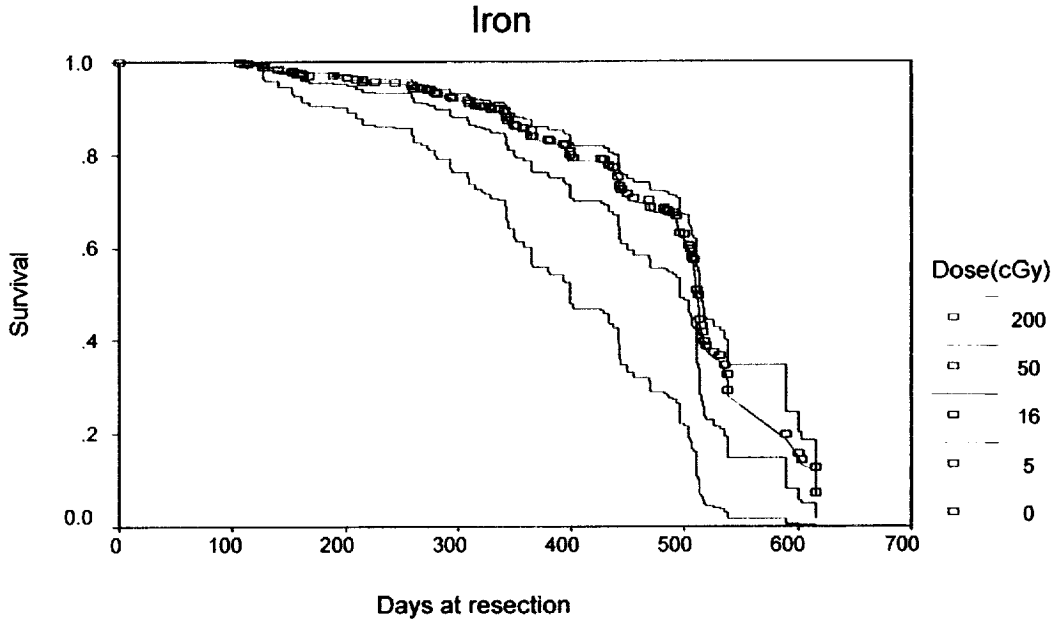


Fig. 26b. Preliminary results of a maximum likelihood analysis for relative risk for tumor-free survival as a function of time after irradiations with iron ions.

10. To establish safe pharmaceutical countermeasures to those risks. **With establishing the magnitude of the health risks from the radiation exposures, this is our most important goal. Although we expected no definitive results until about five years into the study, we are able to report that our initial results show that the risk of mammary cancer arising from exposures of our animal model to HZE particles is definitely reduced (Tamoxifen Project).**

The class of compounds called selective estrogen receptor modulators (SERM's), which includes Tamoxifen, are thought to have outstanding potential for use in estrogen replacement therapy and as chemopreventive agents for a variety of cancers. Burgeoning research and development of new SERM compounds has led to many new and improved SERM's undergoing trials. Tamoxifen, however, remains the prototype SERM for breast cancer chemoprevention, and that was one of the motives for choosing it to investigate the feasibility of using it for HZE exposures. Newer SERM's will hopefully further improve on tamoxifen's effects while reducing its side effects. SERM's are ligands for the estrogen receptor (ER) and modify carcinogenesis in breast epithelial cells by antagonizing ER signaling. However, in other tissues SERM's can act as partial ER agonists and promote the beneficial effects of estrogens in, for example, the skeletal and cardiovascular systems. Interestingly, tamoxifen may also affect carcinogenesis in a number of organ systems by disrupting apoptosis regulation in proliferating cells. In spite of the widespread use of tamoxifen, very little is known about its lifetime effectiveness against radiation-induced neoplasms-particularly those induced by radiation likely to be encountered in space such as protons and heavy ions.

Appropriate animal models provide a powerful means for directly evaluating the effectiveness of particularly promising chemopreventatives against cancers that may occur following radiation exposure. The rat mammary tumor model has been used extensively to analyze the carcinogenic effects of both chemical xenobiotics and physical agents. The Sprague Dawley rat mammary tumor model is particularly well-suited for studies in the low dose range because it is prone to develop mammary neoplasms early in life. Previous studies using the Sprague Dawley model have shown that sublethal doses of radiation (xrays, gamma rays, neutrons-not particularly relevant to space travel) induced mammary tumors, often within one year, and with a linear dose-effect relationship. Thus the Sprague Dawley rat mammary carcinogensis model not only closely resembles human breast cancer biologically, but it also is a highly sensitive model in which to examine the effects of radiation exposure and for testing pharmaceutical countermeasures against radiation effects. Our initial studies have focused on the effects of whole body, low level heavy ion and proton radiation along with chemoprevention of similarly induced mammary tumors using the female Sprague-Dawley rat mammary tumor model. Our rational approach to chemoprevention (SERM's) is based on one of the few successful emerging chemoprevention strategies used in human cancers to date in regard to sporadic or familial neoplasms. The well-studied, widely prescribed, protoypte SERM, tamoxifen has been effectively and safely used in humans for chemotherapy for almost two decades. These advantages, along with an understanding of its molecular mechanism of action suggests it would be an excellent candidate for successful long-term chemoprevention of specific proton and heavy ion-induced cancers. The prospect for successful long-term chemoprevention of this potentially important, late-appearing cancer relevant to space radiation exposure is indeed an exciting prospect.

Finally, we have proposed in the continuation of the Radiation-Effects Program to expand our strategy now that the validity of our initial hypothesis, that drugs can reduce the risk of cancer for low-dose HZE exposure, to include more generic drugs and less toxic drugs including dietary supplements to determine the optimal regimin.

a. To quantitate deletion between direct repeats in progeny of cells exposed to high LET radiation and simulated delta rays. **Modified to align with Team Strategy and to account for reduced funding (DNA Project).**

Four different cell lines were irradiated: F14C-23, 122-2, 3134, and 7#7-7.

1. F14C-23 is human fibrosarcoma cell line with a 122 bp inverted repeat (flanked by direct repeats) inserted into a *neo* reporter gene that is integrated into the chromosome (Kramer et al., 1996). Upon precise deletion of the inverted repeat the cells become resistant to the antibiotic G-418. The spontaneous deletion frequency is 1×10^{-8}, and the deletion frequency after 4 Gy X-rays is about 3×10^{-8}.

2. 122-2, is a human fibrosarcoma cell line with non palindromic insert flanked by direct repeats in the *neo* reporter gene (Kramer et al., 1996). The spontaneous deletion frequency is $<3 \times 10^{-9}$, while the deletion frequency after 4 Gy X-rays is as high as 8.3×10^{-6}.

3. Mouse lymphoblast cell line (7#7-7) contains a 15.3 kb inverted repeat (Akgun et al., 1997). This is a remarkable cell line, in that it contains the longest inverted repeat known to have been cloned. The inverted repeat is very unstable and it may be particularly unstable following exposure to iron particles. The frequency of genome instability is about 0.1 – 0.5 in progeny mice (Akgun et al., 1997). It also shows instability in culture (S. Lewis, personal communication). Genomic instability is detected by a change in the size of restriction fragments resulting from, typically an asymmetric deletion covering the center of the inverted repeat (Akgun et al., 1997).

4. Mouse cell line 3134 is a mammary cancer cell line that contains 200 tandem direct repeats of a 9 kb hormone inducible gene (MMTV driven *ras* gene) (Richard-Foy & Hager, 1987). It was initially obtained for studies on the analysis of changes in DNA supercoiling and topological domain size in this region upon gene expression (Kramer et al., 1999). However, it provides an excellent model system to look at the genomic stability of a 1.8 mb region containing the 200 direct repeats following exposure to high-LET radiation. In collaboration with Dr. Hager's lab we planned to perform FISH analysis for genome rearrangement involving the 1.8 mb region. Metaphase plates hybridized with a *ras* probe reveal a large fluorescent region corresponding to the direct repeat region. The site of integration is near the end of chromosome 4, near a region of telomeric heterochromatin. We also planned analyze the structure of the repeats by pulse field gel analysis as we can examine the entire 1.8 mbp region as well as the structure of the collective 9 kbp repeats.

NOTE: Work on cell lines 7#7-7 and 3134 was not pursued after initial experiments in year #1 at the recommendation of NSBRI external review.

The survival and G-418 reversion frequencies following exposure to 1000 MeV Fe particles at the BNL4 and BNL5 runs have been measured. Following Fe exposure, the rate of G-418 reversion increases as much as a factor of 100.

The survival of four reporter cell lines (122-2, F14C-23, 7#7-7, and 3134) following exposure to 250 KeV X-rays has been measured. All cell lines were sensitive to X-rays in a range that would allow them to be used as reporter cell lines for radiation damage.

The frequency of deletion of an inverted repeat and a nonpalindromic sequence from a *neo* gene in human 122-2 and F14C-23 cells have been measured (by isolating clones resistant to the antibiotic G-418) following exposure to 250 KeV X-rays.

The nature of the reversion events, which involve precise deletions between direct repeats, has been analyzed by PCR analysis.

The rate of reversion or the mutant frequency for the deletion mutations in the *neo* gene have been calculated for control and X-ray exposed cells. The frequencies are about 1-2 × 10^{-7} in sham (non irradiated) cells. The rate increases by as much as a factor of 60 following exposure to X-rays.

We have successfully cloned a 770 bp perfect inverted repeat (2 x 385 bp Alu sequence) and a 763 bp inverted repeat with a 39 bp nonpalindromic center (2 x 362 bp + 39 bp) in *E. coli*. This has taken considerable effort as this is 6-7 times longer than any inverted repeat we have previously cloned. A number of modifications to existing protocols had to be developed to get this. We are putting *Sph*I adaptors on it to clone it into the *neo* gene in pJJ999 (the vector be used for electroporation into rat cells.

Survival and G-418 reversion in cell lines 122-2 and F14C-23 following exposure to 1000 MeV/n Fe (results from BNL-4 & BNL-5).

Drs. Sinden, Braby and Ford went to Brookhaven National Labs and irradiated many samples of the 4 cell lines. Irradiation involved only frozen samples of F14C because the spontaneous mutation rate is so low that we may not have been able to detect many revertants irradiating samples on T25 dishes (applying selection to irradiated cells prior to growth and expansion of the culture). Cell lines 122-2, 3134, and 7#7-7 were irradiated both as frozen stocks and as living cells attached to T25 dishes or grown in suspension (7#7-7).

On May 10-11, 1999 three cell lines (122-2 subclone A, 122–2 subclone B, and F14C-23) were treated with with 0, 25, 50, and 100 cGy 1000 MeV Fe. These cells were treated attached to T25 flasks. The first set of plates (for all 3 cell lines) has been expanded and is under selection for G-418 reversion. Cells were also plated for survival analysis. The survival analysis is nearing completion. Cells from two other sets of plates were frozen for analysis at a later date. Also exposed to Fe were large samples of F14C-23 (thirty two 150 mm dishes) exposed in suspension. These cells survived nicely and have also been frozen for subsequent analysis. These experiments are critical as they represent a repeat of the experiment form a year ago. Moreover, during BNL5 all cells were exposed either on plates or in suspension. (No frozen cells containing DMSO were exposed.)

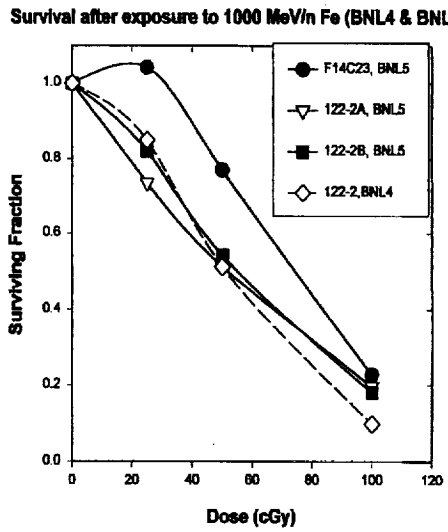


Fig. 27: Survival following exposure to 1000 KeV/n Fe particles.

Figure 27 shows survival data for cells exposed to 1000 MeV/n during the BNL 4 & 5 runs. We were successful in measuring survival at doses equitoxic to those for the X-rays. G-418 selection data for one experiment for cells treated on plates during the BNL-4 run are shown in Figure 28. A reasonably good







dose response was observed

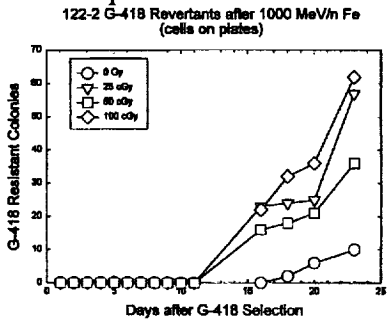


Figure 28: G-418 resistant revertants following 1000 MeV/n Fe.

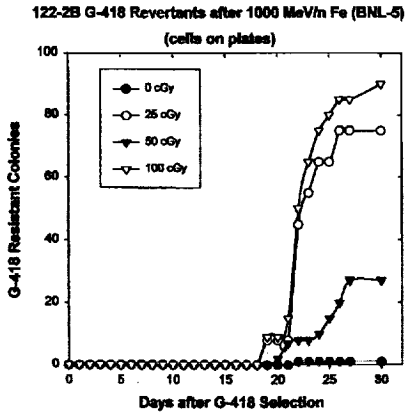


Figure 29: G-418 revertant cells; selection after first passage.

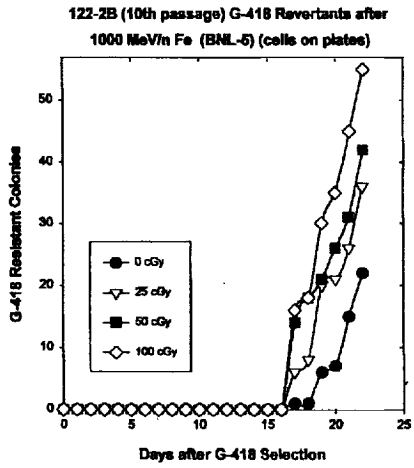


Figure 30: G-418 revertants after 10th passage of growth following exposure to 1000 MeV/n Fe.

following heavy iron treatment. The results for cells irradiated as frozen cultures during the BNL-4 run were not encouraging; G-418 revertants were not strongly dose dependent and few revertants were detected. For this reason we decided to only treat cells on plates or in suspension for the repeat of this experiment during BNL-5. Results from BNL-5 G-418 selection are shown in Figure 29. Cells were also grown for 10 passages before selection was applied and these results are shown in Figure 30. These selections have been repeated several times. The results, to date, indicate that the instability associated with G-418 reversion in cell line 122-2, which involves deletion of the 122-2 bp non palindromic mutation insert occurs in a dose dependent fashion, although the response has shown some variability. The selection following growth for 10 passages simply indicates that the G-418 resistant cells persisted during unselected growth. In addition. we have isolated individual clonal cell lines that are still G-418 sensitive and then applied selection to determine if the cells, after recovery from DNA damage, continue to show a high level of genome instability. Revertants were rare in these analyses.

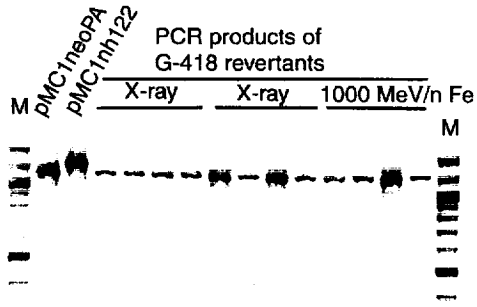


Figure 31: PCR analysis of deletion of the 122 bp insert in the *neo* gene.

To analyze the nature of the mutation leading to G-418 resistance, individual G-418 resistant revertant colonies were picked and cloned, and then the DNA was purified for PCR analysis of the region of the neomycin gene containing the 122 bp mutation insert. It is expected that G-418 resistance will result from precise deletion of the mutation insert, as observed previously for spontaneous reversion (Kramer et al., 1996). Figure 31 shows representative results for a few G-418 resistant revertants from 122-2 cells following X-ray and Fe-particle exposure. The size of the PCR product is consistent with that for the original *neo* gene (lane pMC1neoPA), not that for a gene containing the 122 bp mutation insert (pMC1nh122). It is important to note that the original 122-2 cell line contains only a single integration of the *neo-hyg* gene construct (Kramer et al., 1996). Southern analysis of the G-418^r cell lines is planned to look for genome rearrangement, as observed previously (Kramer et al., 1996) (and to confirm the deletion) but these analyses have yet to be completed.

b. To quantitate deletion between direct repeats in cells adjacent to those irradiated by high LET particles and or simulated delta rays. **Not done because of reduced funding (DNA Project).**

c. To quantitiate deletion between direct repeats in cells adjacent to those irradiated by high LET particles and or simulated delta rays, i.e., in "bystander cells." **Not done because of reduced funding (DNA Project).**

d. To develop additional reporter constructs to increase the spectrum of mutational events that can be quantitated. Specifically, constructs that report deletion, recombination, and gene conversion events will be developed. **Modified to align with Team Strategy and to account for reduced funding (DNA Project).**

Dr. Sinden's group has constructed and purified the Alu inverted repeat constructs for introduction into human and rat cells. They have been successful in cloning a 758 bp perfect inverted repeat made of two 379 bp fragments containing the consensus human *Alu* sequence. In addition, they have cloned a 753 bp inverted repeat consisting of two 358 bp Alu-containing fragments flanking a 37 bp non palindromic center. These sequences are six times longer than the longest inverted repeat we had made to date and it represents a considerable achievement as inverted repeats are notoriously unstable in *E. coli* (see for review (Sinden, 1994)). This was done by a two step cloning procedure introducing the *Sac*I-*Pst*I fragment containing the *Alu* consensus sequence from plasmid pPD39 (Batzer et al., 1994) into pUC19. The *Sac*I-*Eco*RI fragment was then introduced creating the perfect inverted repeat. For the quasipalindrome, the *Xba*I-*Pst*I fragment was first introduced into pUC19. As seen in Figure 32, plasmid AluP-7 contains a plasmid of the correct size as well as an insert consistent with 758 bp. A plasmid band corresponding to the parent pUC19 is also seen. This is consistent with deletion and, although this occurs, sufficient non deleted plasmid can be purified for the next cloning step. Plasmids containing the 753 bp quasipalindrome (with the 37 bp non palindromic center) are considerably more stable as seen in the lanes labeled AluQP-7 to AluQP-10 where the ratio of the insert-containing to parental (insert-deleted) plasmid is higher.

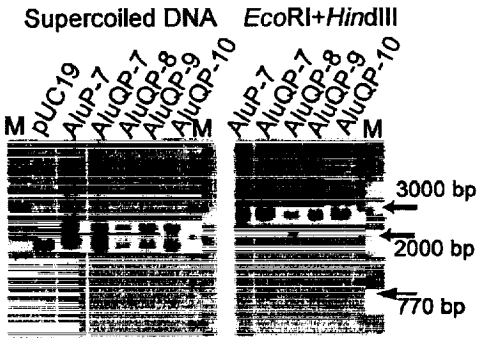


Figure 32: Analysis of Alu inverted repeats cloned into pUC19. Supercoiled DNAs (upper band in insert-containing plasmid while the lower band represents deletion products, uncut plasmids; EcoRI + HindIII, plasmid cut with those enzymes to generate the 770 bp inverted repeat. AluP-7, strain containing the perfect inverted repeat; AluQP-7-10, clones containing the inverted repeat with the 37 bp non palindromic center.

This is a very important result as it demonstrates that we can work with this inverted repeat in bacteria. This facilitates construction of the reporter cell lines. It is important to note, however, that the construction of the human cell lines could be done without cloning these plasmid in *E. coli.*

II. RISK REDUCTION ACHIEVED BY PROGRAM

The infrastructure for the NSBRI was designed with the research team as a mission-oriented, focused group. Because of the limited amount and high cost of beam time for radiobiological studies with HZE particles,.collaborating and sharing resources have been essential to achieve the goals of Institute and the team. The Core Project has acted as the central focal point not only the irradiations but also the team effort. NSBRI External Advisory Council cited the need for us to examine antioxidants, methyl donors, and/or other "nutriceuticals" which might have radioprotective effects. It further encouraged us to extent the chemoprevention studies to the lifetime of the animals, which we did early in the last award period, and to consider alternate animals and cell models, which we have done in our new proposal. This project has required the coordination of several

subproject with multiple groups working at different accelerators and institutions simultaneously and with other projects within the Radiation Team The level of effort and structure is more equivalent to that of NIH program grants, rather than the required RO1 format. The daily housing charges for our existing animal colony of 2000 animals, before doing any additonal experiments, is $0.52 per rat per day not including indirects and overhead. This corresponds to over $600,000, well beyond the average award. Acknowledging full well the financial and programamatic impact of large-scale animal experiments at low doses, we propose that this program be considered for five-year funding cycles rather than three. Such a time scale would be in line with the time periods required to execute and complete these types of studies and is the norm for program grants at NIH.

An organizational chart showing the major research sub-projects we proposed for future studies is presented. In the spirit of the directives we received from the NSBRI reviews, we are using the Min mouse to examine intestinal cancers. Dr. Bert Vogelstein and Dr. Kenneth Kinzler at Johns Hopkins, eminent scientists in this area, have agreed to consult with us and their letters are included in the Appendix. We will evaluate Sulindac with the Chemoprevention Project and Bowman-Birk Inhibitor and other dietary supplements in collaboration with Dr. Ann Kennedy at the University of Pennsylvania, a co-investigator and a leader in this field.

Again, responding to the suggestions of the External Council, we will be working with Dr. Francis Cucinotta's group from NASA JSC to do PCC and three-color and M-FISH studies. Dr. Jerry Williams is moving toward retirement, so he is not submitting a renewal for the Cytogenetics Project. He has agreed to provide his technical staff, Dr. Yonggang Zhang and Haoming Zhou, so we can continue to provide the irradiated cells and tissues to our colleagues for the cell biology.

Because the potential level of CNS damage, perhaps the last poorly evaluated risk, remains largely unknown, we will work with Dr. Marcelo Vazquez at BNL to evaluate our animals for these types of effects.

We had intended to provide tissue and cell samples and collaborate with Dr. Christopher Lange at SUNY Downstate Medical Center, supporting his work to examine the kinetics of double-strand breaks (DSB) using pulsed-field gel electrophoresis and with Dr. Barry Rosenstein at Mt. Sinai Hospital in NYC to examine TP53 mutations. However, their proposals are not among those under consideration for funding, so we are reorganizing to be certain that there are are strong basic biological data being generated, which is crucial for extrapolating the animal results to humans.

It is essential that we bridge the gap from rodent to human and apply our results to adequately assess the radiation risks to personnel in space as rapidly as possible. We have joined forces with Dr. Andrei Yakovlev's epidemiology and biostatistics group at the University of Utah to analyze and model our results. Dr. Zaider will work with us further improve the comprehensive risk assessment for the difference environmental scenarios in space.

Our main objective was to provide the biological systems and scientific base, so the hypothesis that pharmaceuticals could be used to reduce the risk of relevant diseases, particularly carcinogenesis, could be evaluated. In a rat model with a natural sensitivity to mammary tumors, we were able to establish the basic risks for carcinogenesis in a radiation-sensitive tissue found in humans and in a different species other than the mouse harderian gland examined earlier by Alpen et al (1993). We now have a good data base with this model, and more data will becoming forth over the next three years from our existing animal colony which have been irradiated with various scenarios during these last two years. At the same time, we examined the cellular and subcellular characteristics of these mammary cells, the corresponding human mammary cells, and other cells for other relevant tissues (colorectal, leukemia,

and lymphoma). Because we have a good data base with this model, we proposed two additional experiments be done, one during this three-year cycle (sequential irradiations) and one during the subsequent three-year period (protracted fractionations). At the recommendation of the NSBRI External Review Council, we are moving toward returning to the mouse model and a tissue with tumors having minimal hormone stimulation. For that reason, we have chosen the Min mouse and intestinal tumors as our new endpoint. Colon cancer was chosen because colon cancer is one of the major tissues contributing to the effective doses to be received by astronauts in space. Moreover, recent advances by Dr. Ann Kennedy's group at the University of Pennsylvania and others in radiation therapy have resulted in specific relatively nontoxic pharmaceuticals that reduce the risk of late cancers. Further, these drugs work primarily by reducing oxidative stresses, making them less dependent upon the specific tumor type, while retaining the desirable characteristic of working in the promotion and progression stages of the disease.

The hypothesis to be tested is that drugs are effective in reducing the risk of intestinal cancers arising from proton and HZE exposures at lower, protracted doses relevant in space travel. Most animal models used to examine colorectal cancers use drugs to stimulate sufficient tumors, making them unsuitable for our needs. The Min mouse, recently developed at Johns Hopkins and other institutions, is a non-transgenic mutation that is hypersensitive to the development of intestinal tumors, with typically a dozen or so occurring naturally within the first six months of the life of the mouse. Bowman-Birk Inhibitor (BBI) and other oxidative-stress reducers already been shown to be an effective chemopreventive agent for colon cancer in this model, so the hypothesis to be tested is that such drugs are effective in the case of proton and HZE exposures at lower, protracted doses relevant in space travel. To test this hypothesis, we are proposing to irradiate Min mice with photons, protons, and heavy ions with the same dose regimens used in our previous studies (i.e., 50 to 500 cGy with protons and photons and 5 to 50 cGy with 1-GeV iron ions).

The overall goals, then, are:

1) We will continue to completion our experiments presently underway;
2) We will irradiate one new cohort of animals, preferrably theSprague-Dawley rats because we have a good data base, with protons and iron ions to examine the synergistic effects of sequential irradiations with different particle species (graduate thesis partially funded from two other grants);
3) A portion of this cohort could be examined for CNS damage;
4) We will irradiate Min mice with photons, protons, and iron ions to establish and the relative biological effects and differences in responses to the three types of irradiations;
5) We will examine changes in the risks of intestinal cancers in the Min mouse resulting dietary supplements of BBI;
6) We will examine the chromosomal changes in the tissues using M FISH techniques;
7) We will analyze the data to establish mechanistic pathways and theoretical models for extrapolating from animals to humans and from the accelerator radiations to the complex radiation environment in space.;
8) We will theoretically determine response functions for the different animal results and new human results from Hiroshima and Nagasak,i and we will calculate the risks for humans in typical radiation fields in space;
9) We will supply animals, cells, and tissues to projects for other principal investigators, including Dr. A. Kennedy (U. of Penn) and Dr. M. Vazquez (BNL) in addition to our work with Drs. F. Cucinotta's group at the NASA Johnson Space Center).

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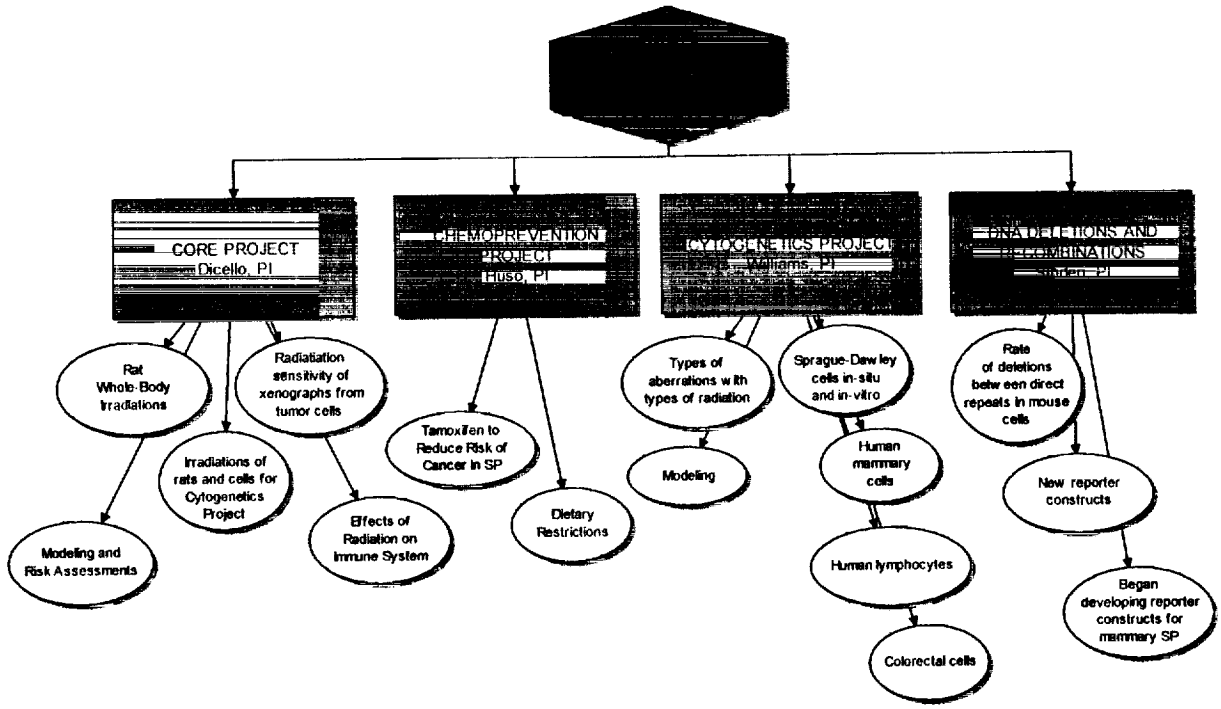
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APPENDIX A

DATA AND FIGURES FROM TEXT

Research Structure for the Radiation Effects Team



An outline of the organizational structure and research accomplishments of the Radiation-Effects Team.



Fig. 1. One of the systems for irradiating animals at the Brookhaven National Laboratory.

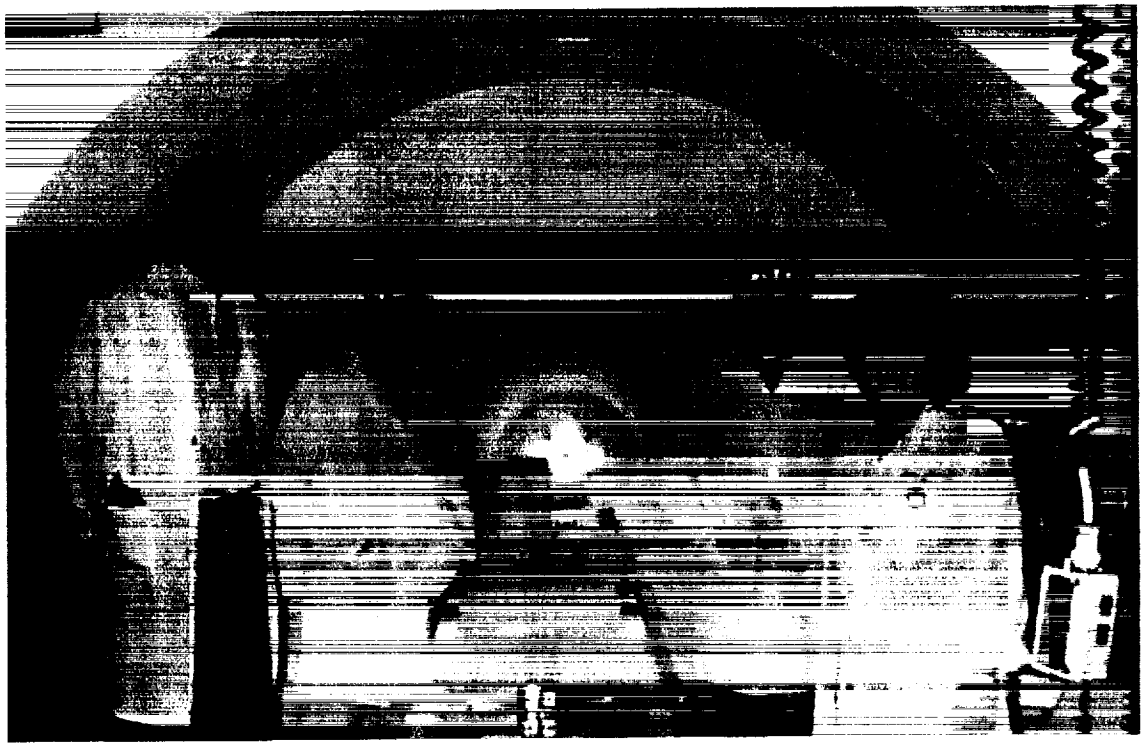


Fig. 2. One of the apparatuses for irradiating cells at the Loma Linda University Medical Center.







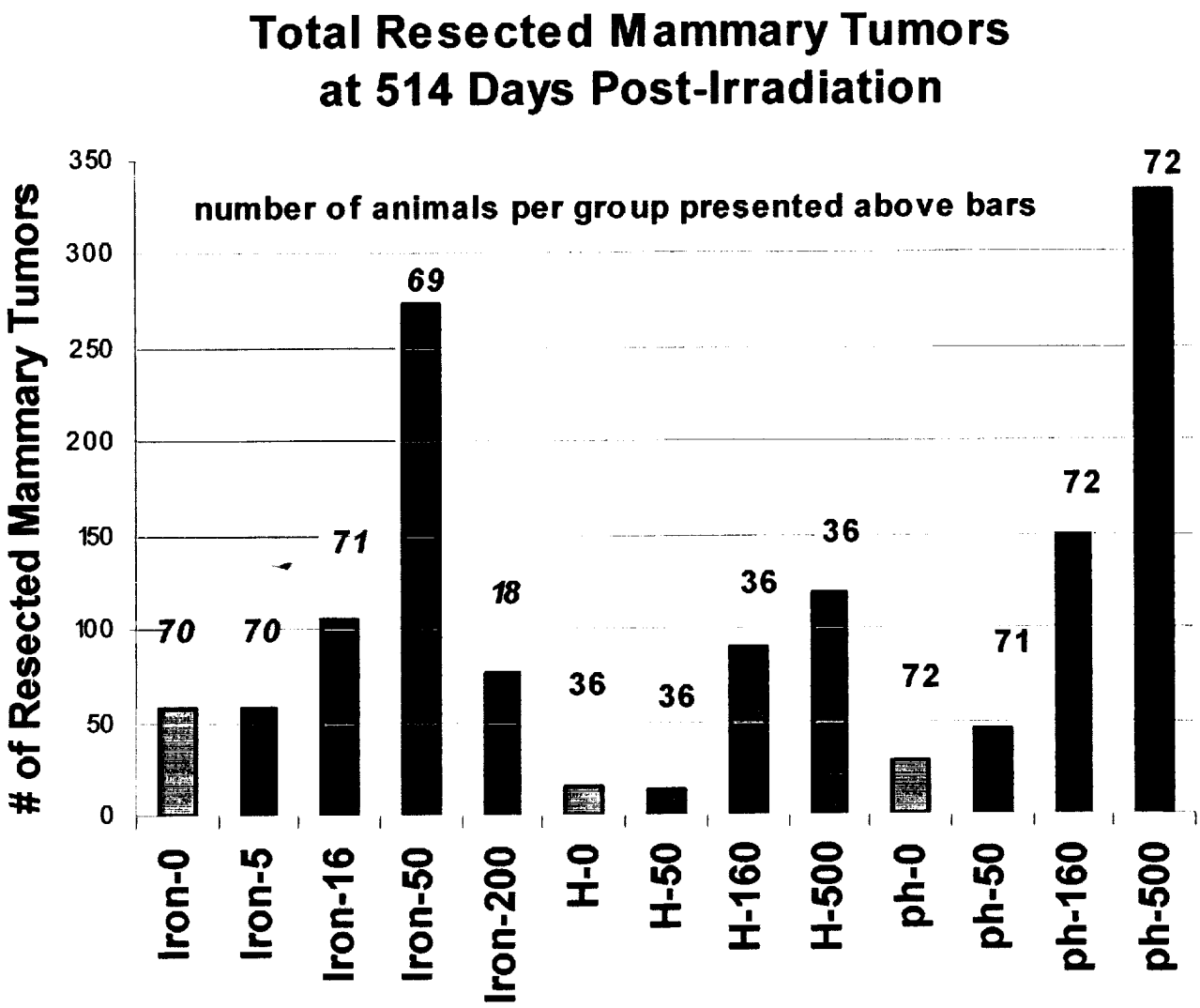


Fig. 3. The number of excised tumors observed as a function of radiation type and dose.

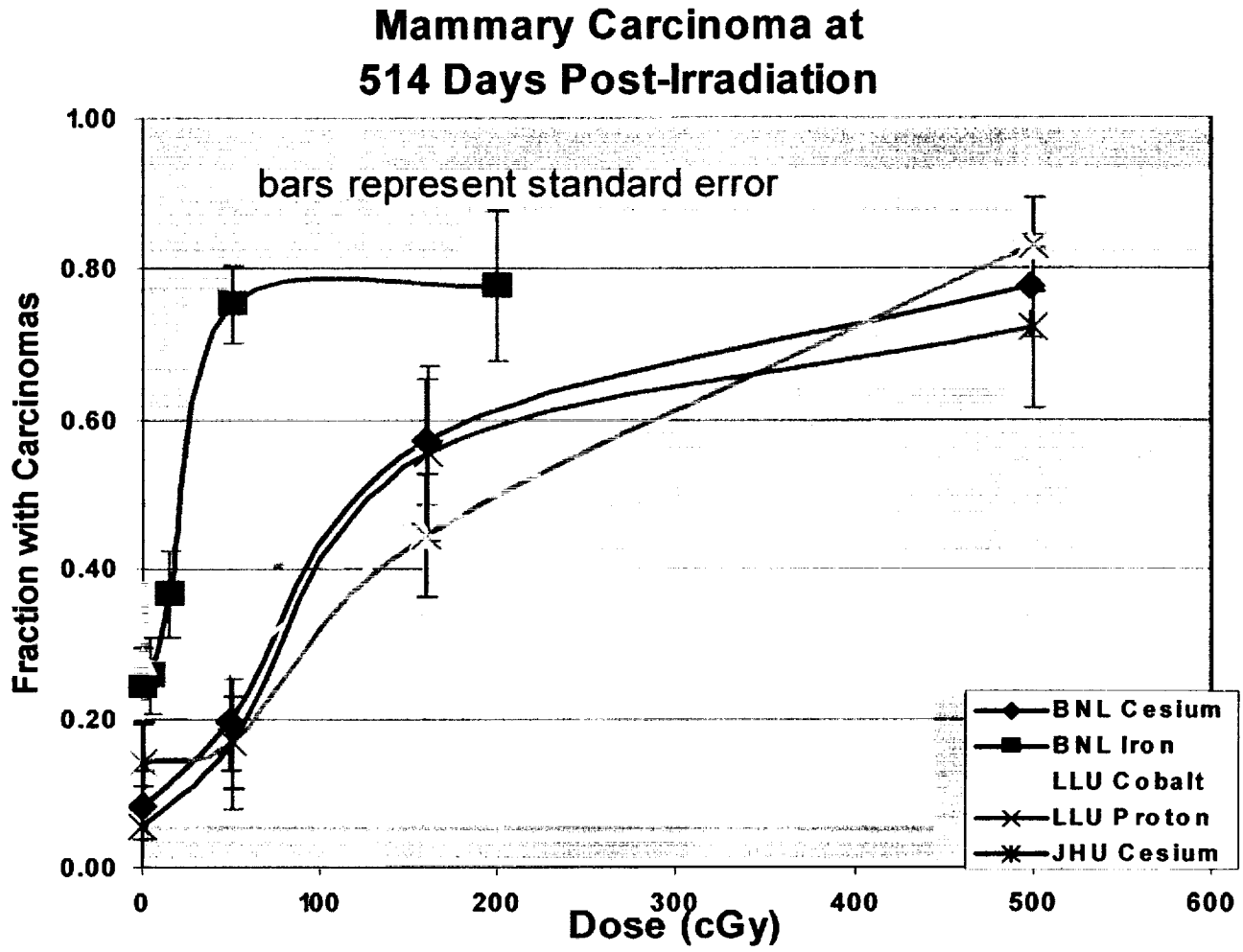


Fig. 4. Fraction of mammary carcinomas observed in each group at 514 days after irradiaton.

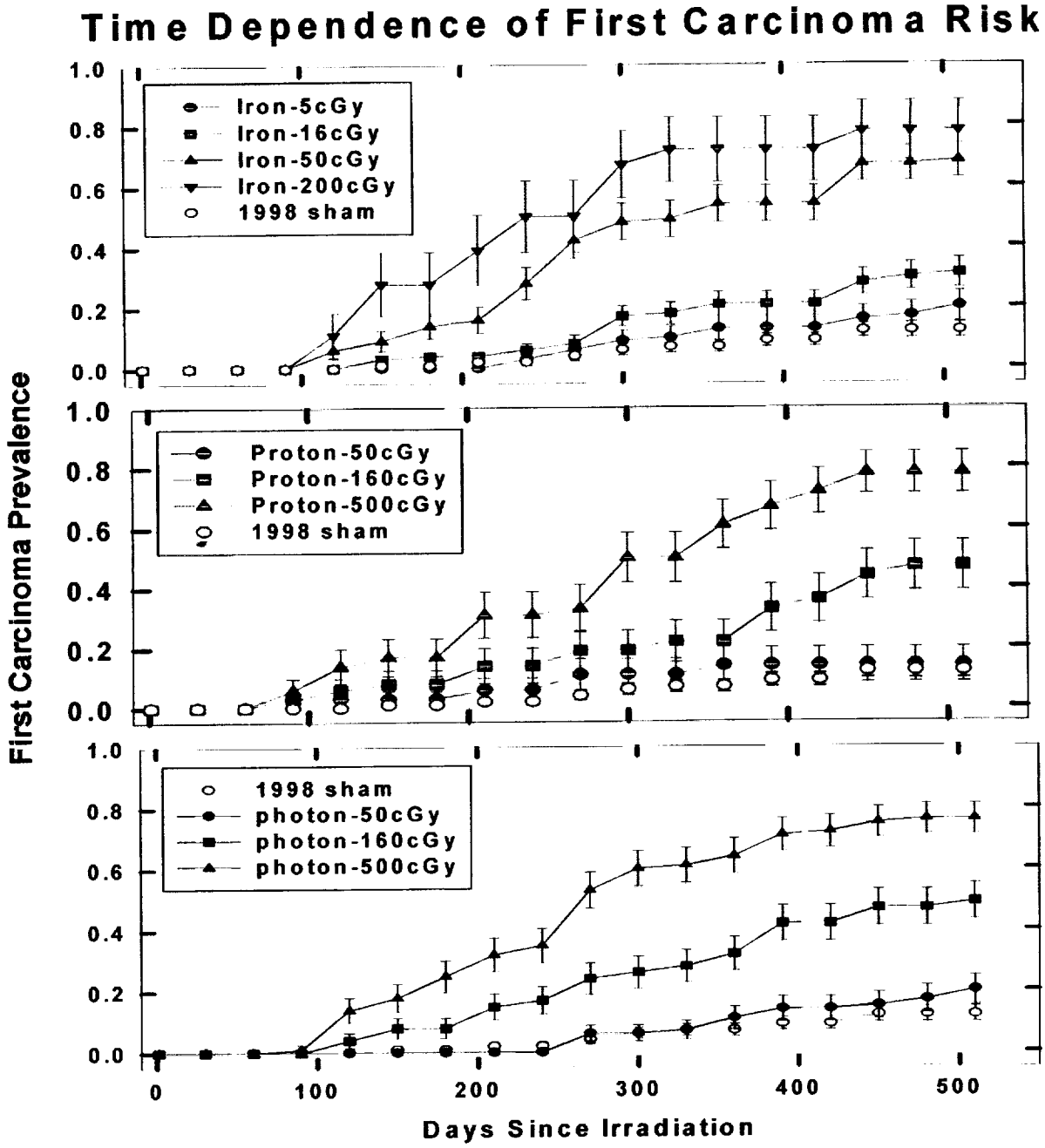


Fig. 5. Prevalence of first carcinoma as a function of time after irradiation.

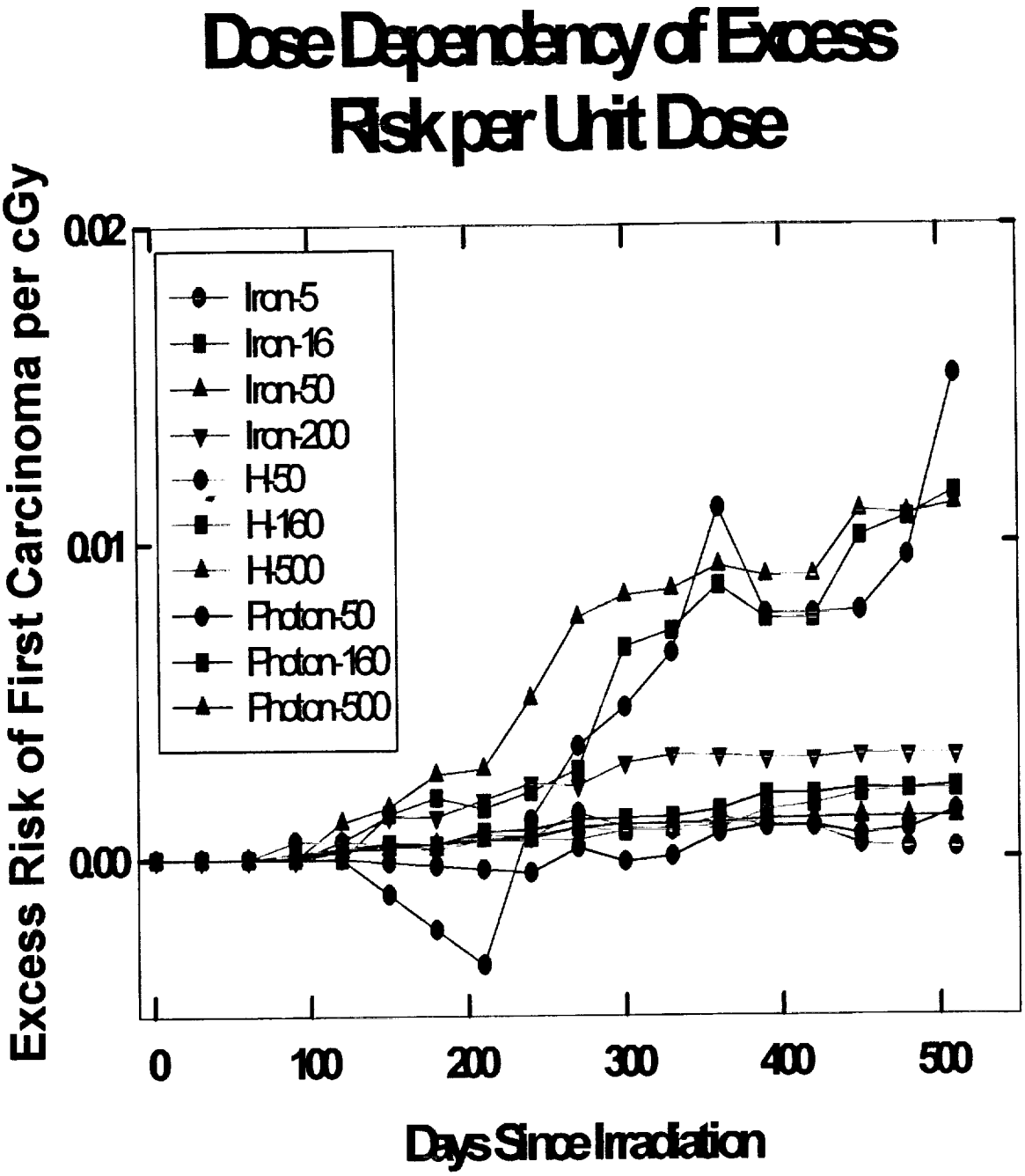


Fig. 6. Excess risk per unit dose.

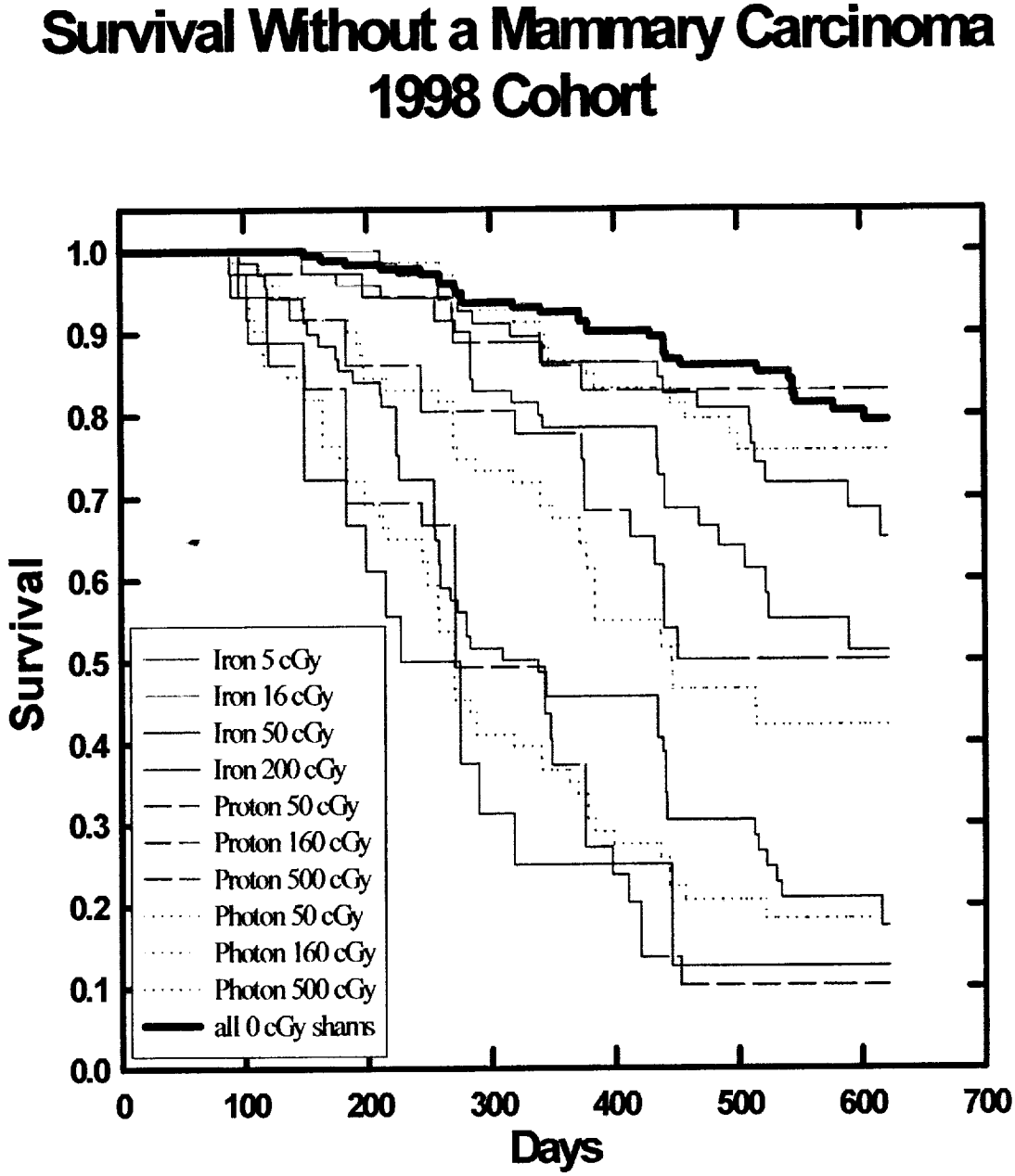


Fig. 7. Results of a Kaplan-Meier survival analysis.







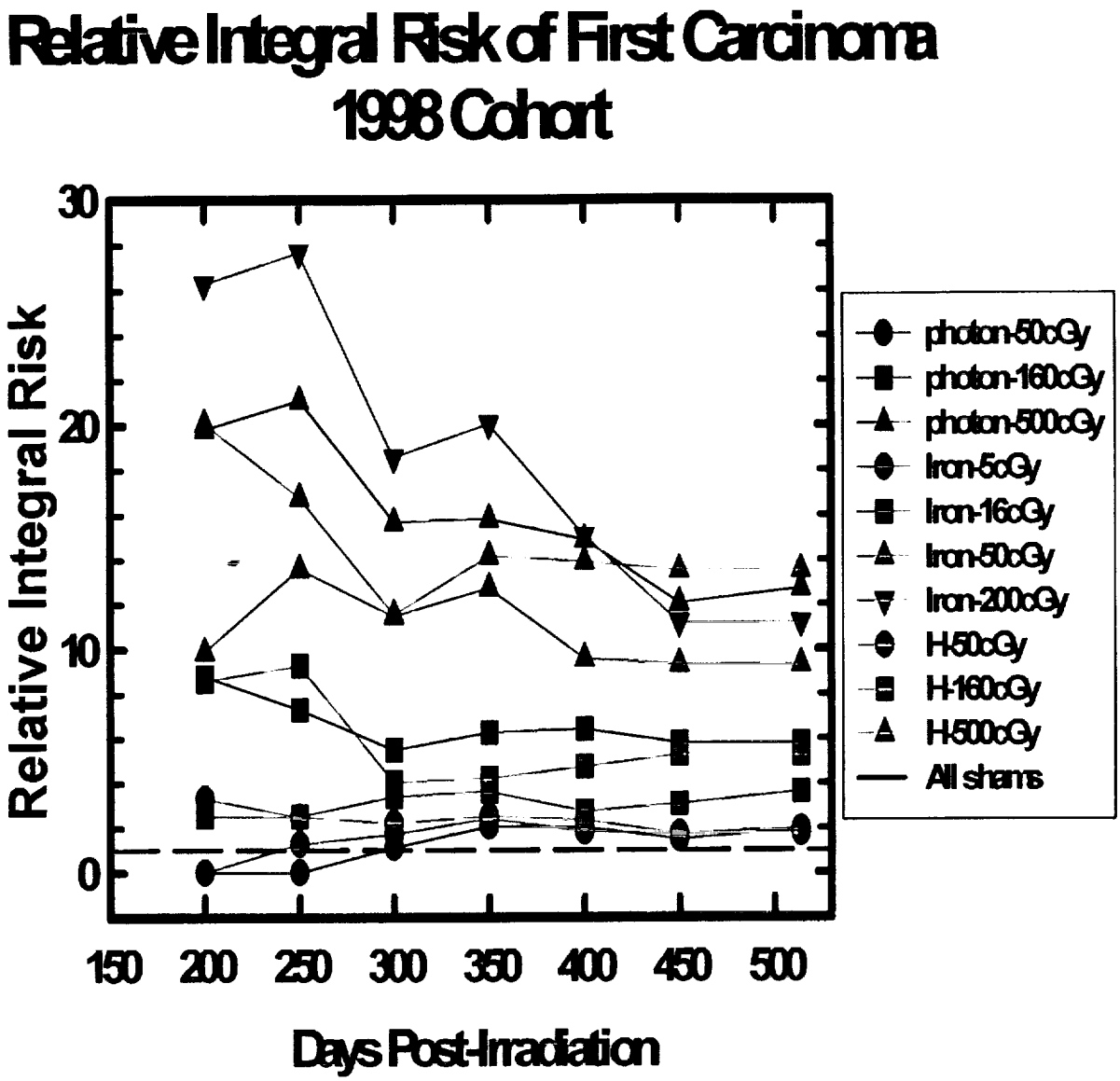


Fig. 8. Relative risk as a function of time since irradiation.

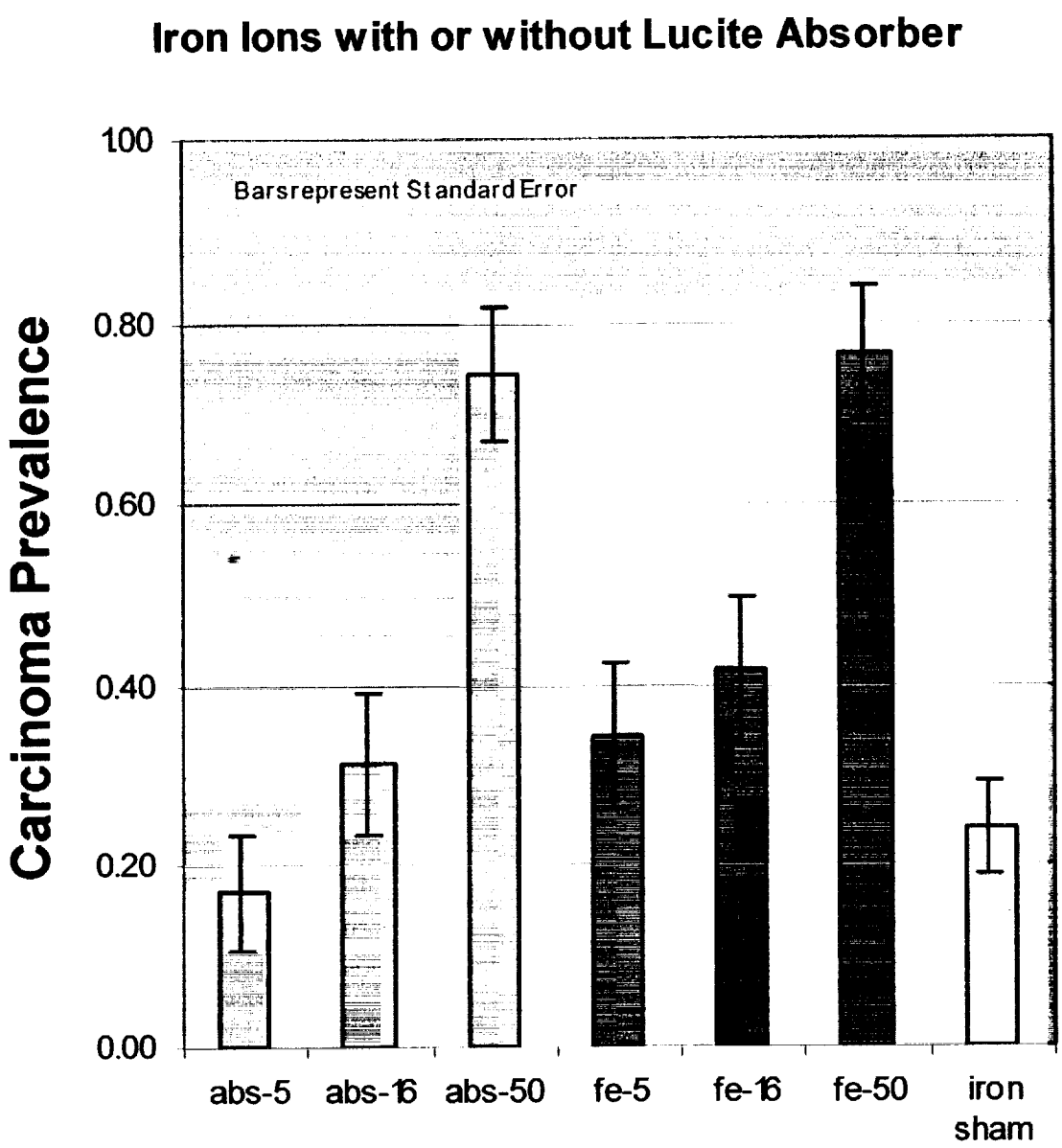


Fig. 9. A comparison of prevalence with lucite placed upstream of the rat during irradiation.

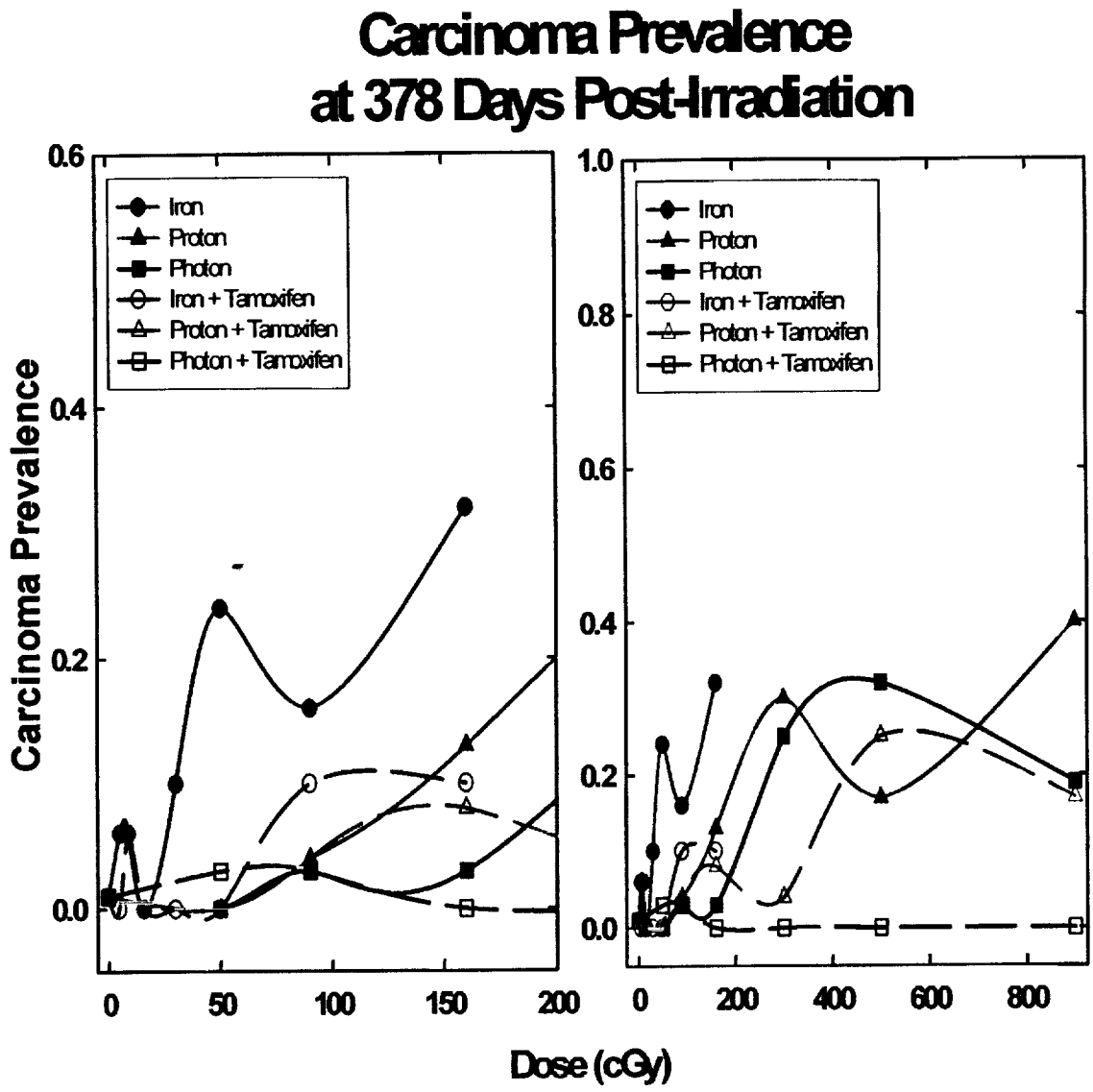


Fig. 10. Prevalence of cacinomas with and without subsequent Tamoxifen.

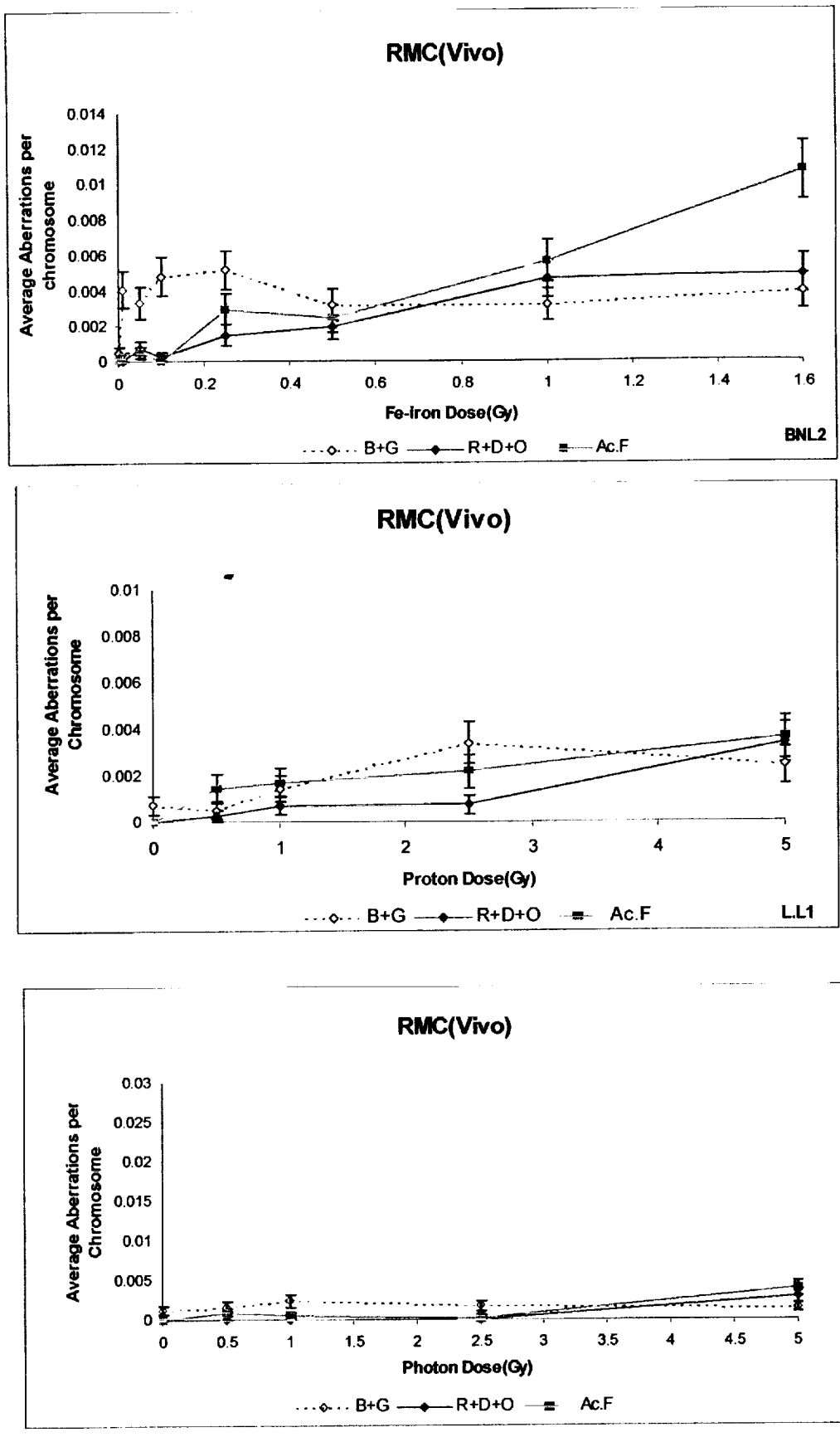


Fig. 11. Average aberrations per chromosome in rat mammary cells as a function of dose for iron, protons, and photons.

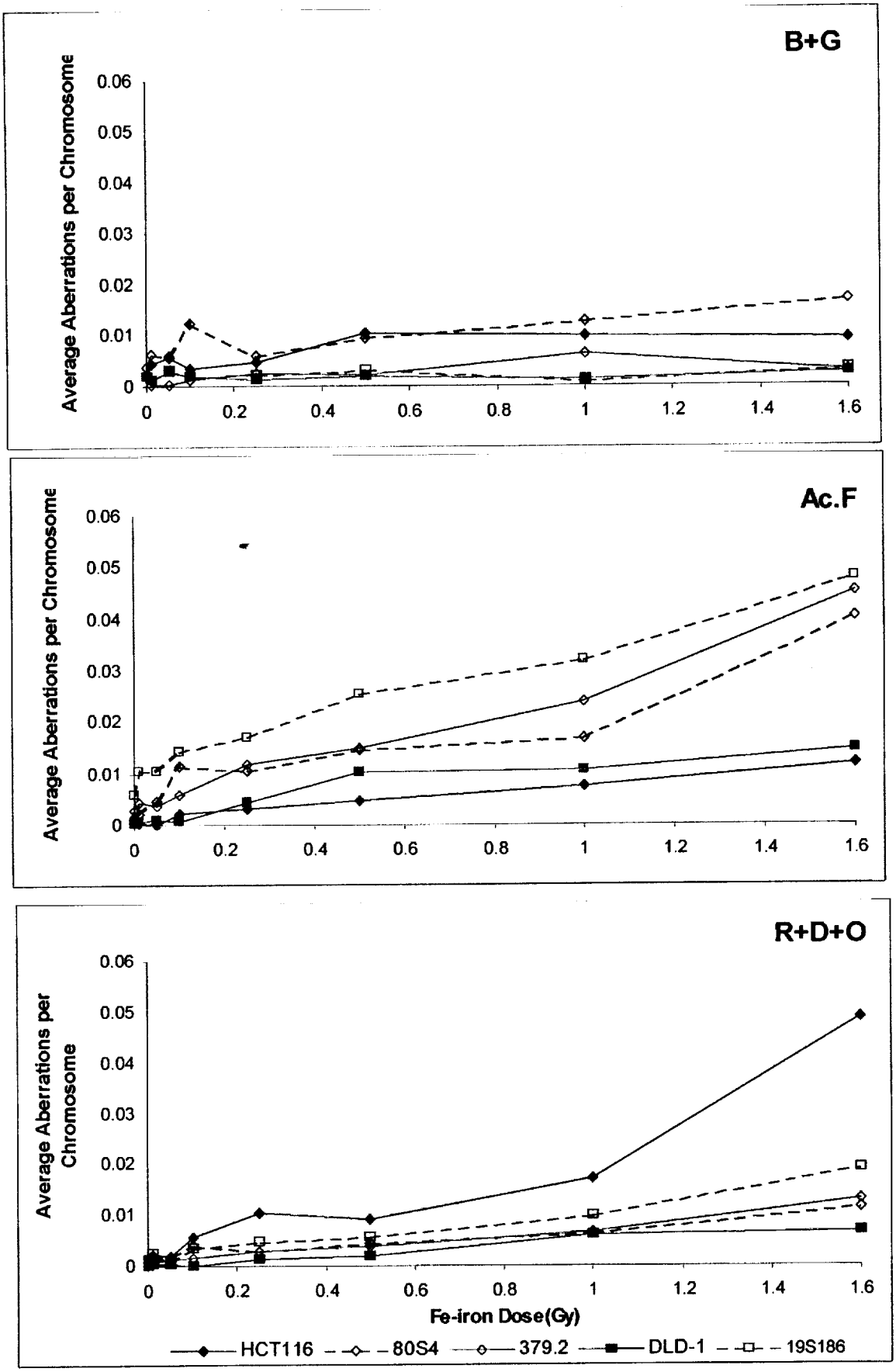


Fig. 12. The average aberrations per chromosome versus dose for the three types of aberrations.







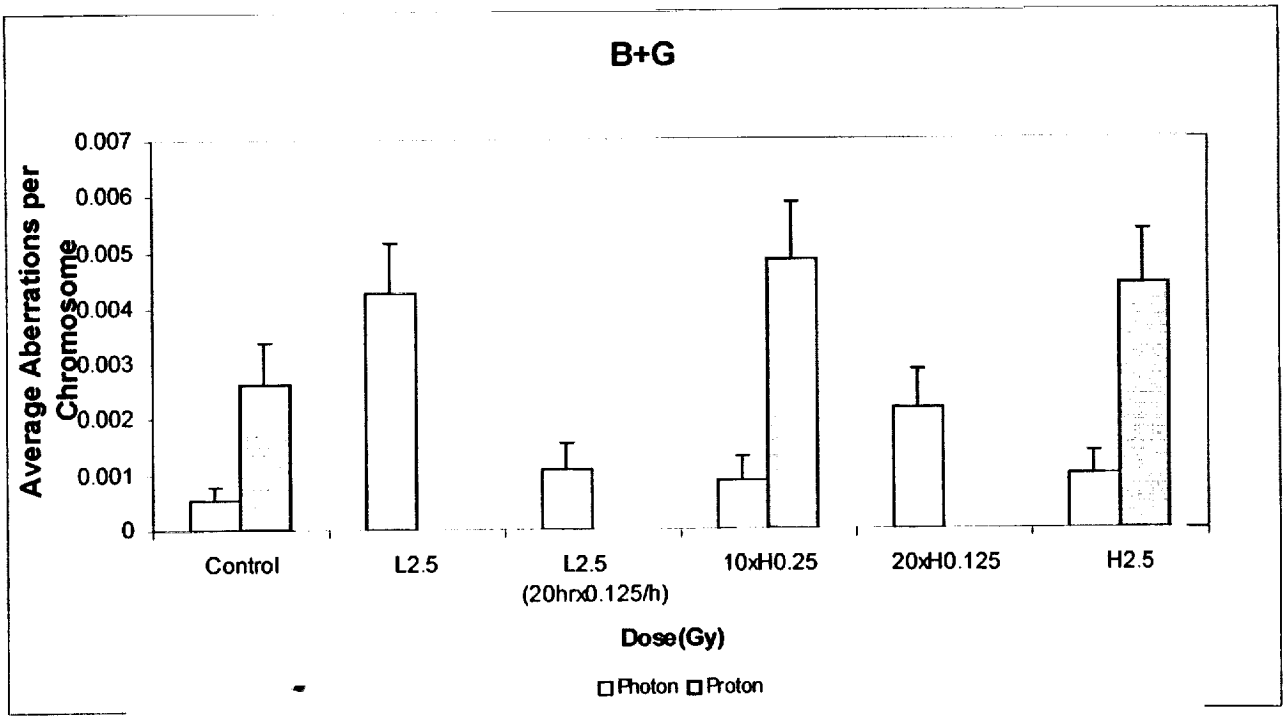


Fig. 13. A

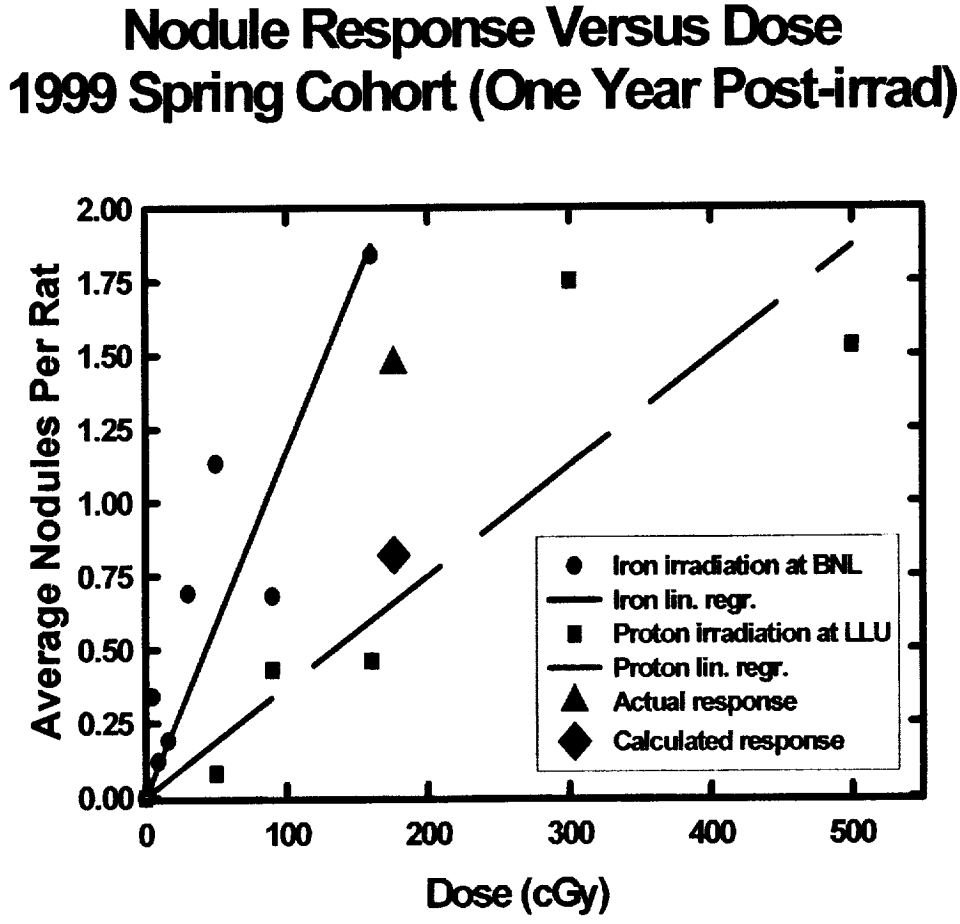


Fig. 14. A comparison of measured response for sequential irradiations with iron ions and protons with the expected additive.

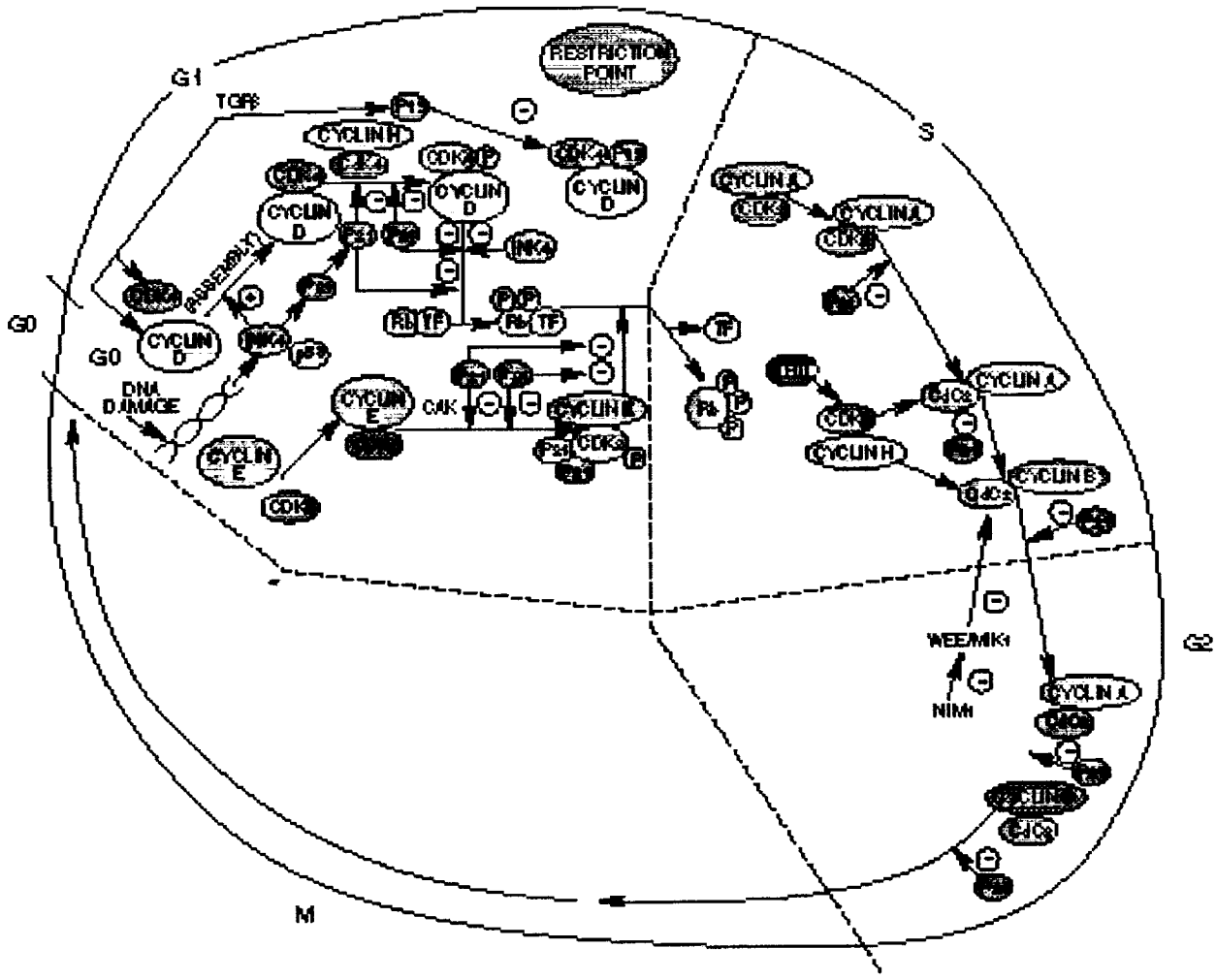


Fig. 15. Diagram illustrating functional roles of cell-cycle regulation proteins (Adapted from Hirama and Koeffler, 1995).

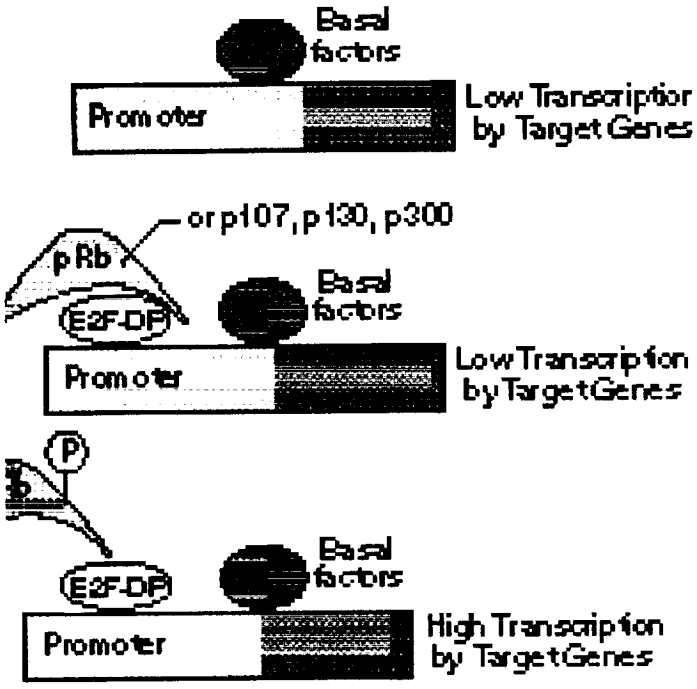


Fig. 16a. Diagram illustrating role of pRb and its phosphorylation and expression of transcription factors E2F/DP.

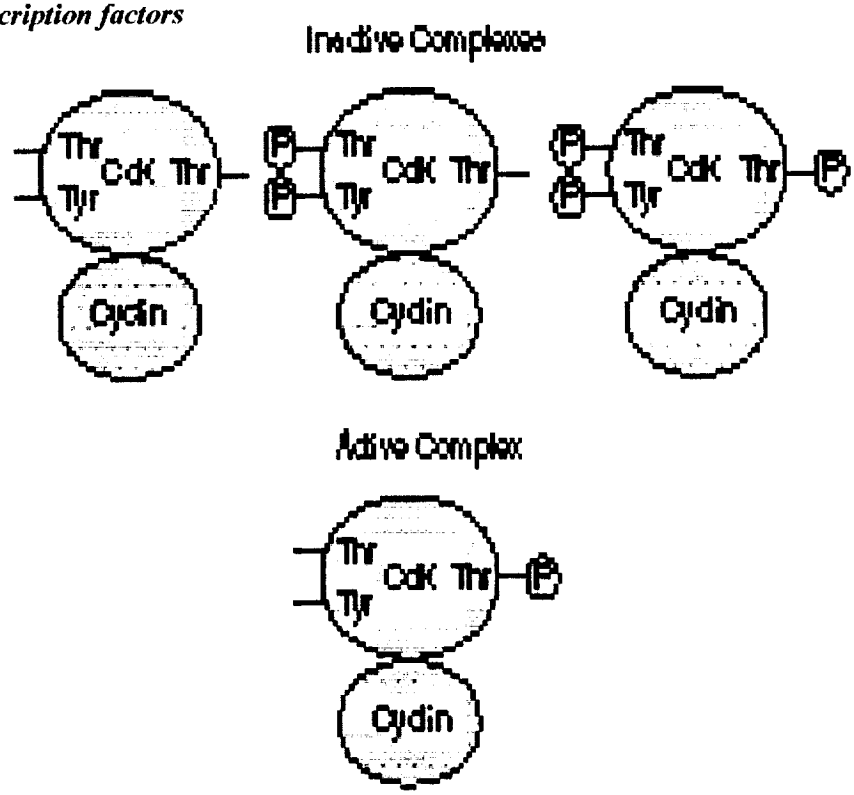


Fig. 16b. Diagram illustrating positive and negative controls on kinase activity of cyclin/cdk complexes.

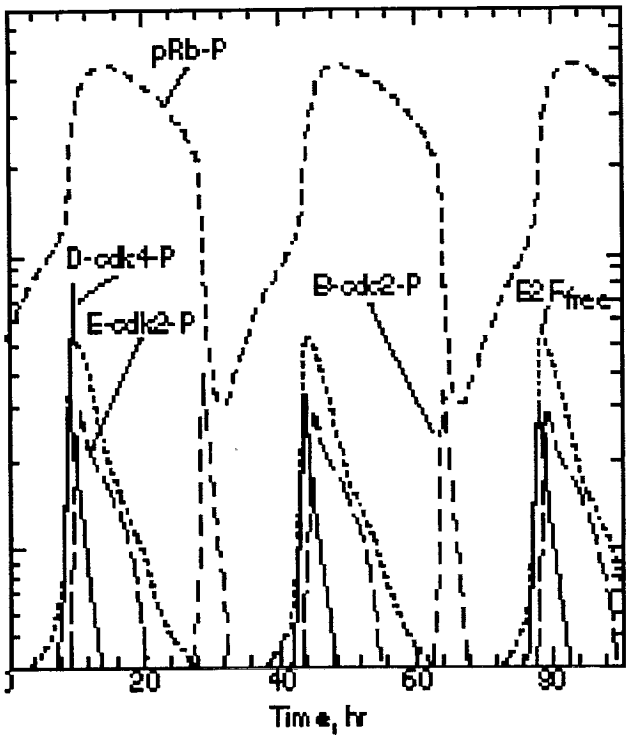


Fig. 17a. Calculations of cyclin E kinase in normal and Rb- cell lines showing proliferation (shortened G1 times).

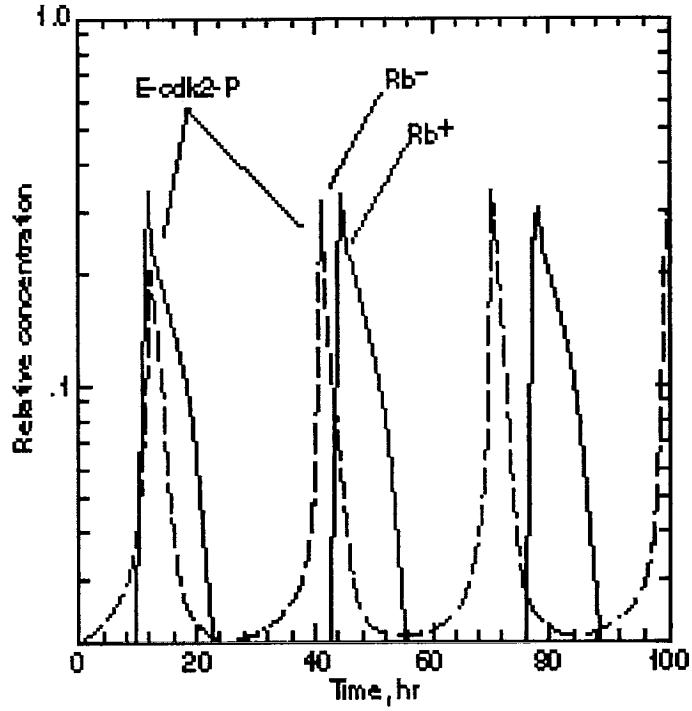


Fig.17b. Calculated percent expression of the cyclins D, E, and B as a function of time over several cell cycles showing limit cycle behavior found in the model.

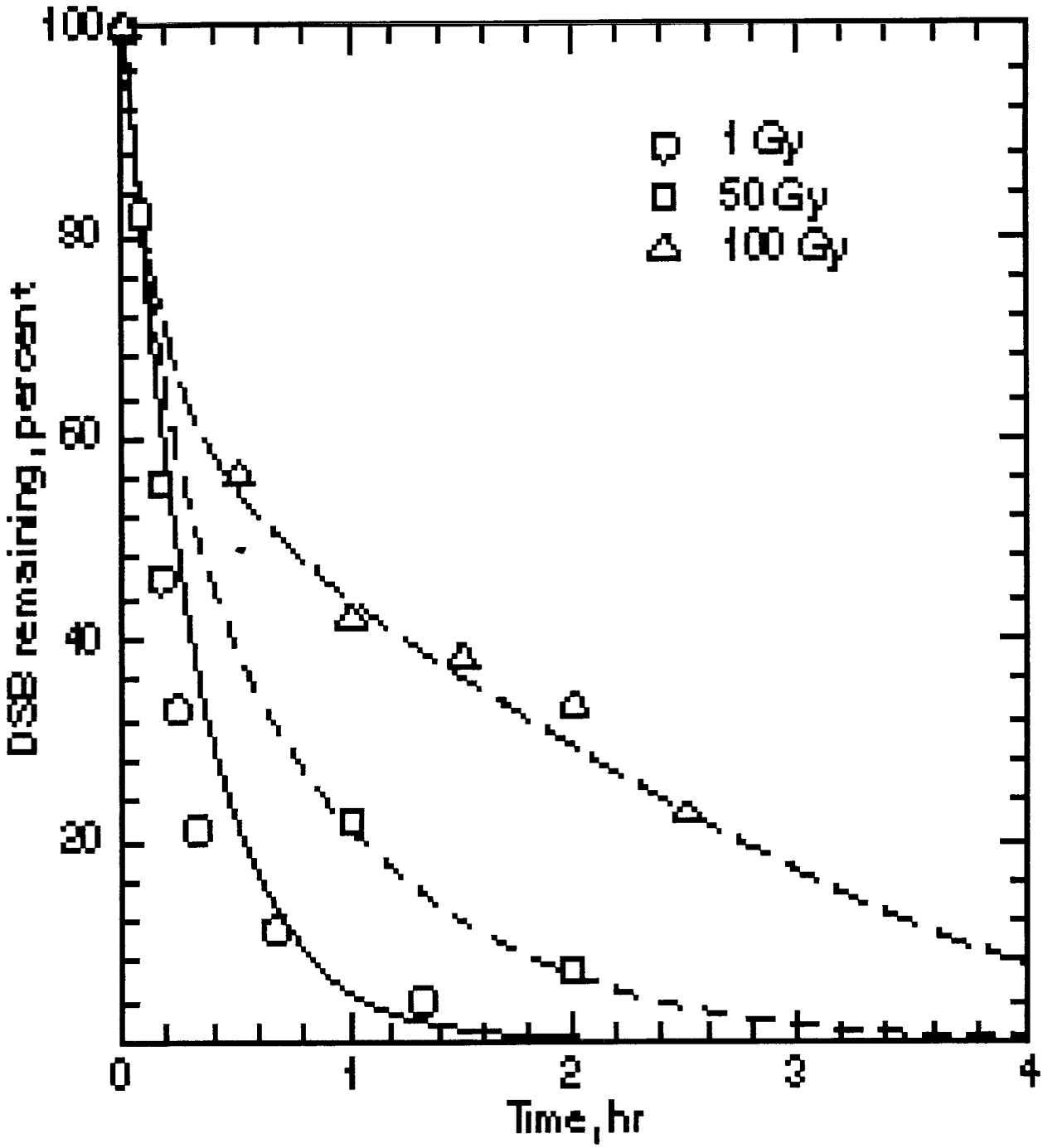


Fig. 18. Calculations of DSB repair versus time after exposure. Also shown are DSB repair data of McWilliams et al., 1983, and G. P. Van Der Schans et al., 1983.

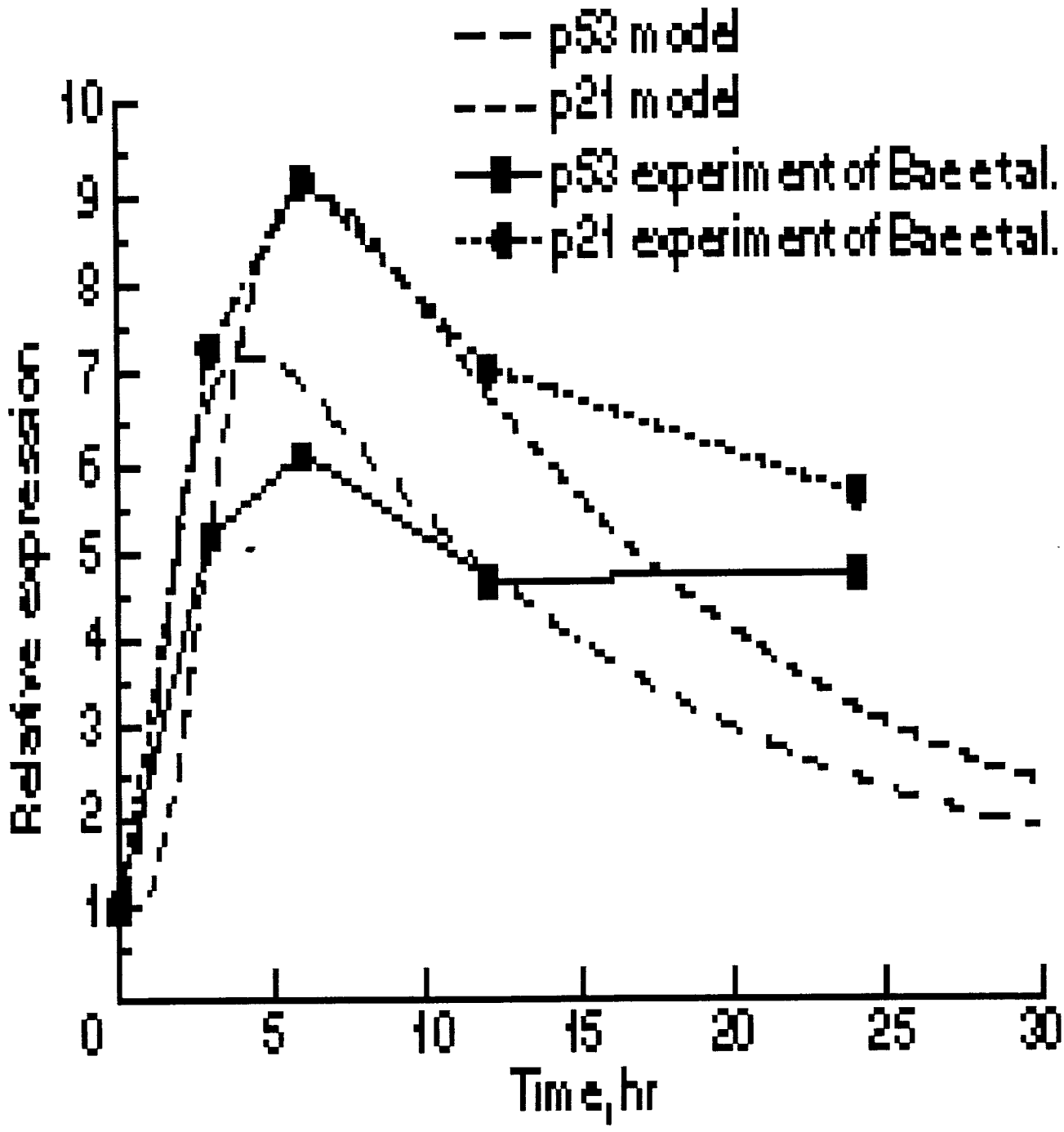


Fig. 19. Model calculations of p53 and p21 expression versus time after 6.3 Gy acute exposure as fit to data of Bae et al., 1995.

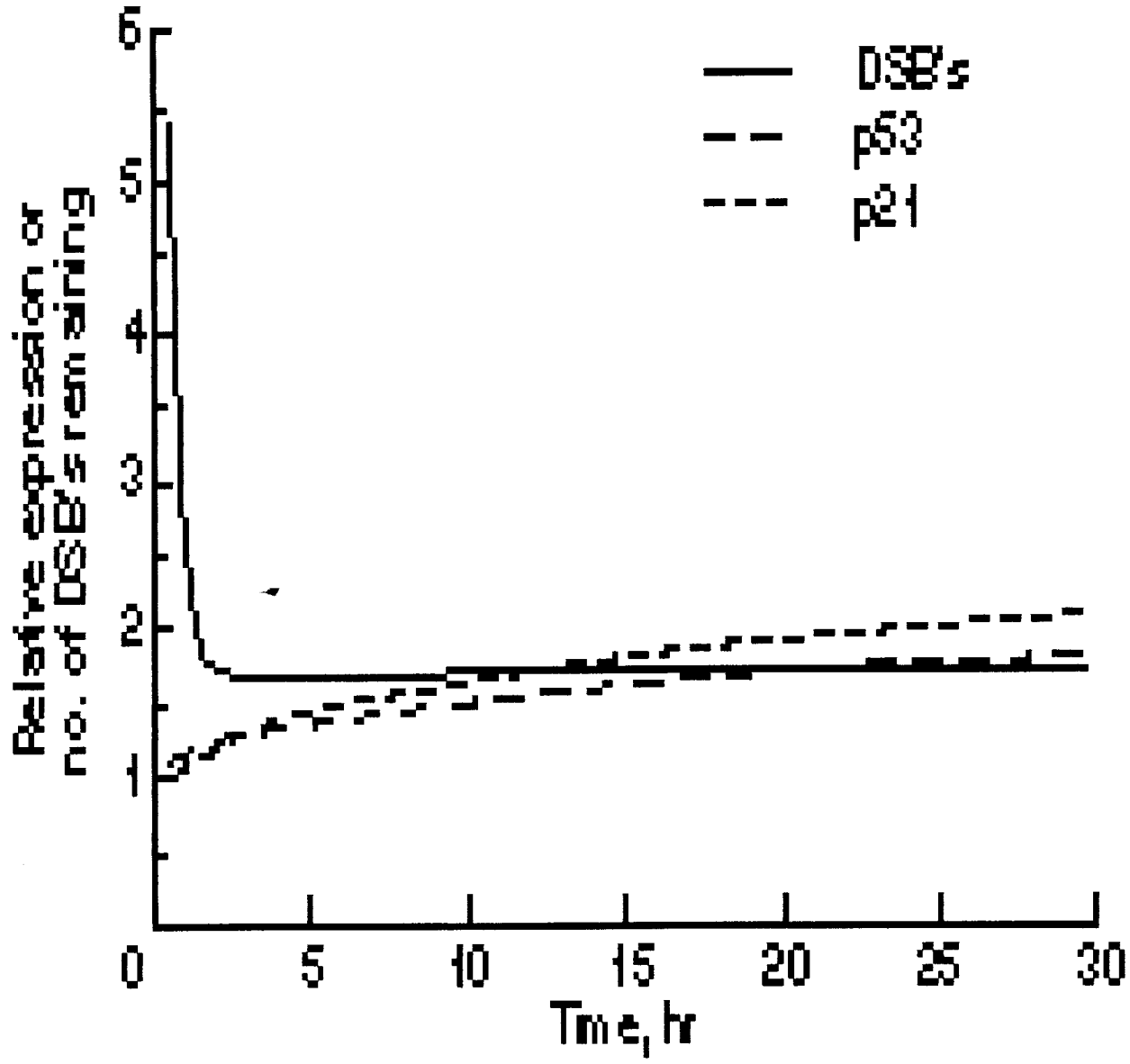


Fig. 20. Predictions of model for number of DSBs remaining and relative expression of p53 andp21 for 0.1 Gy/hr gamma irradiation.

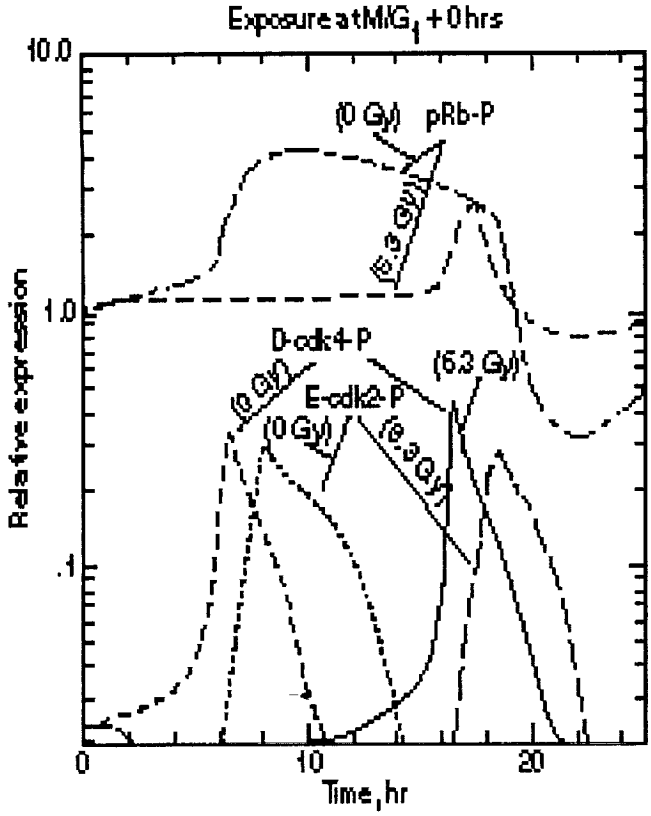


Fig. 21a. Calculations of relative expressi several proteins after acute exposure of 6.3 M/G1 border.

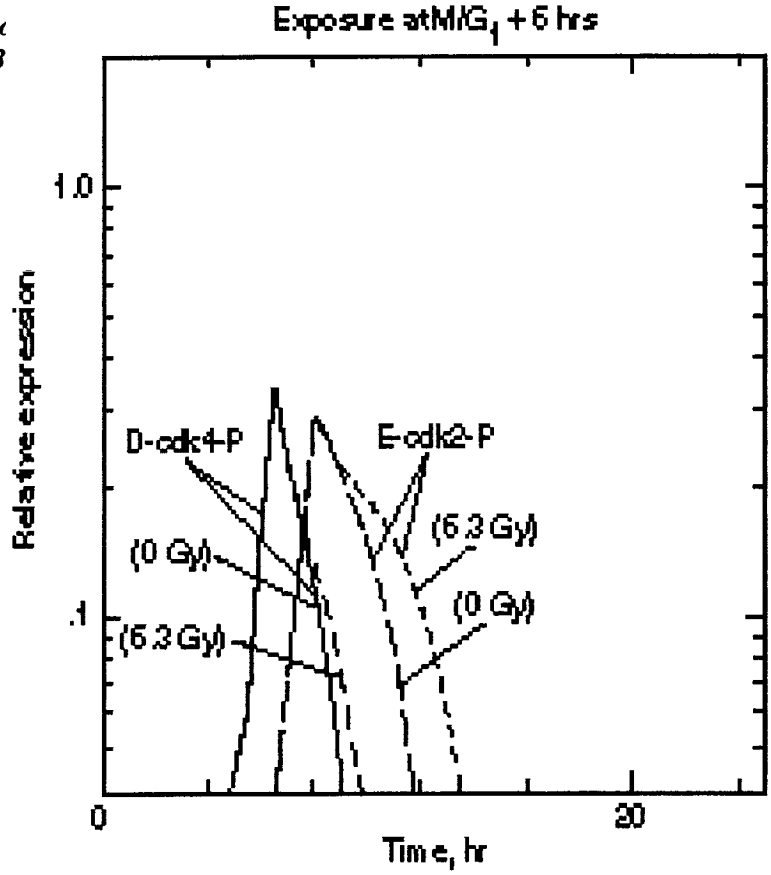


Fig. 21b. Same as Fig. 8a except it is exposed at M/G1 border plus 6 hrs.

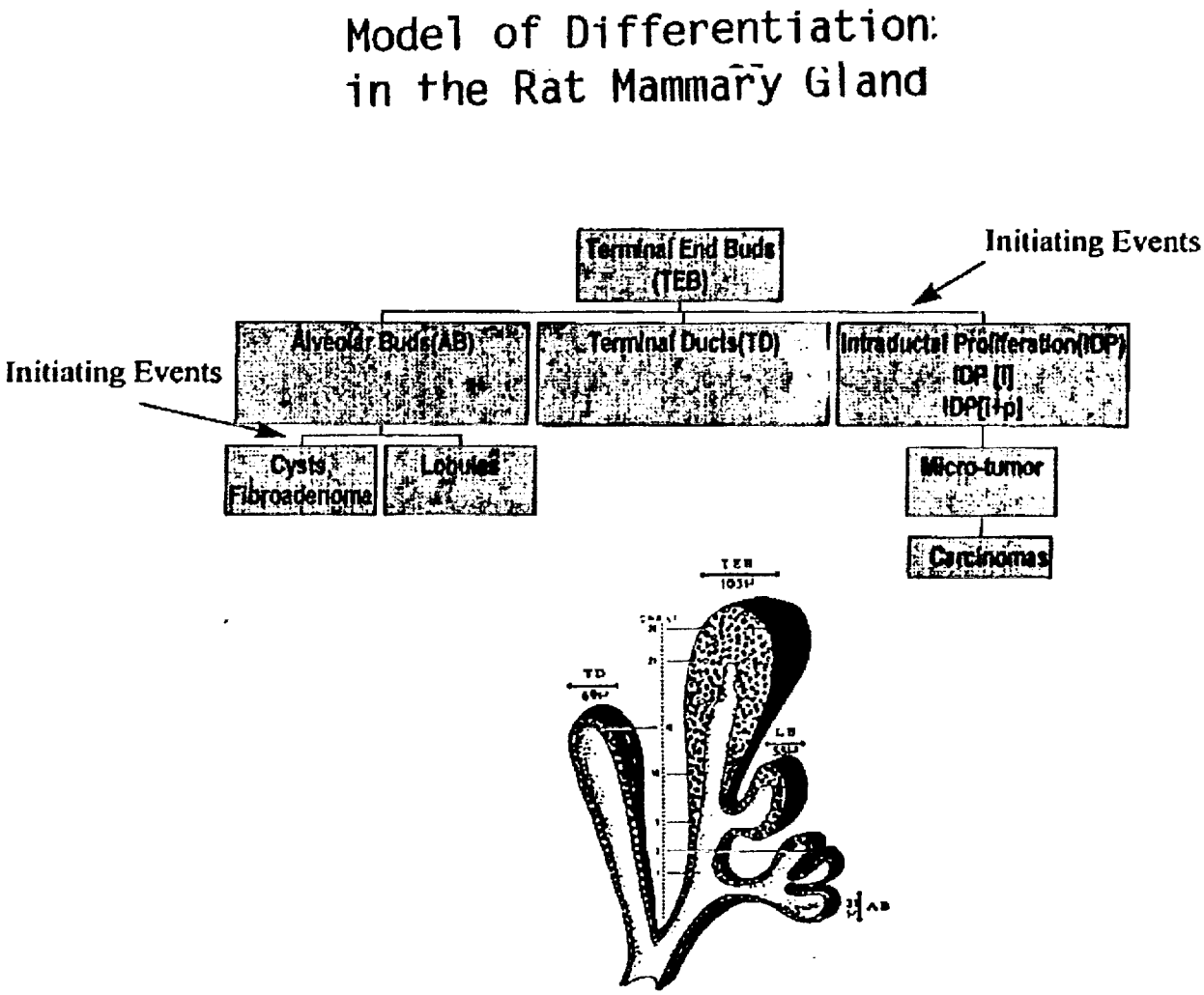


Fig. 22: A schematic of the pathways underlying the mathematical model used to simulate mammary-tumor induction in the Spraque-Dawley rat, according to the paradigm of Russo et al., (1983).

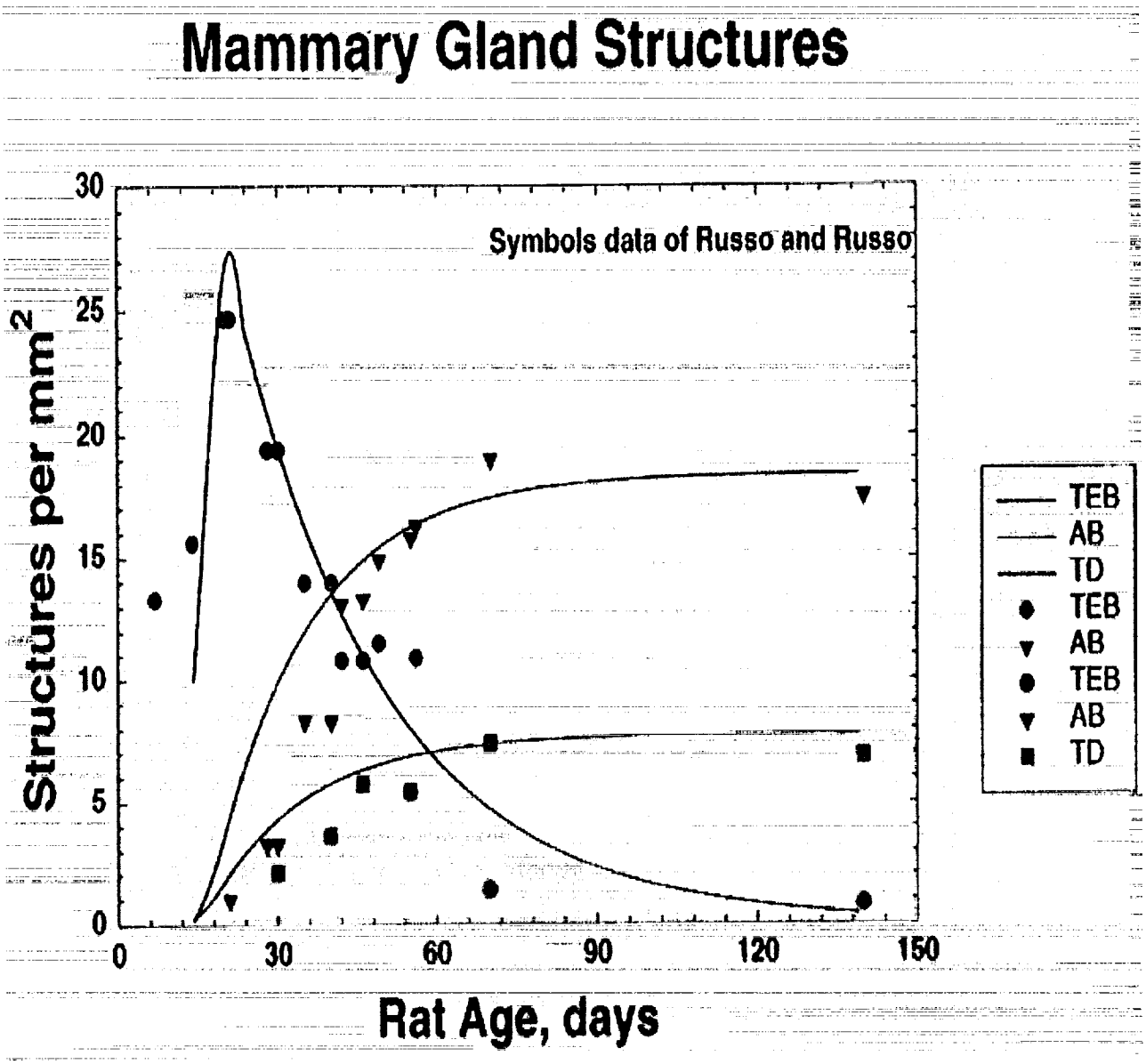


Fig. 23: A comparison of the theoretical model (solid lines) with the experimental results (symbols) for the progression and regression of differentiation of the rat mammary gland for the terminal end buds (TEBs), the alveolar buds (ABs), and the terminal ducts (TDs).

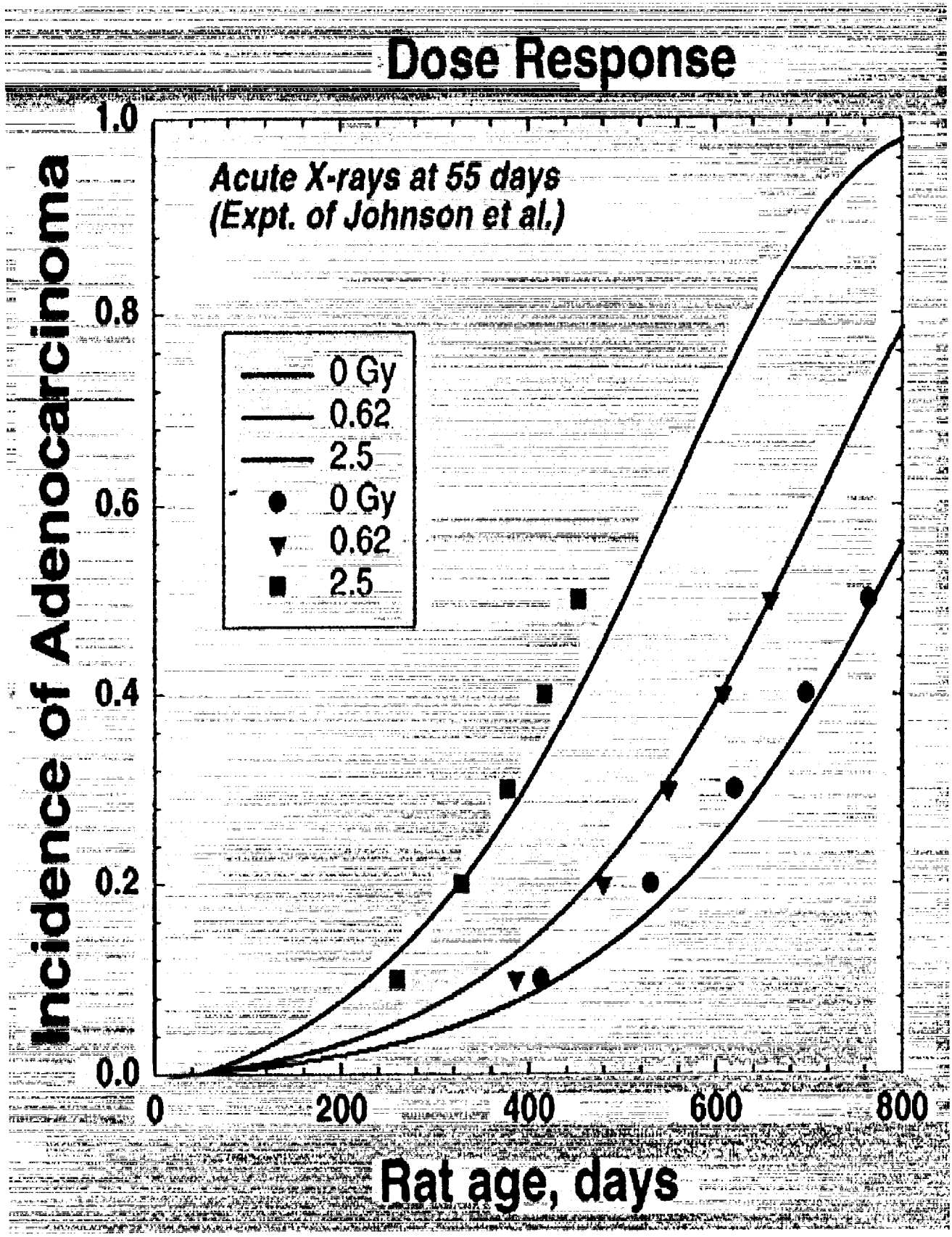


Fig. 24: A comparison of the incidence of adenocarcinomas in the rat mammary gland for the theoretical model (solid line) and the experimental results (symbols) of Johnson et al. 1989).

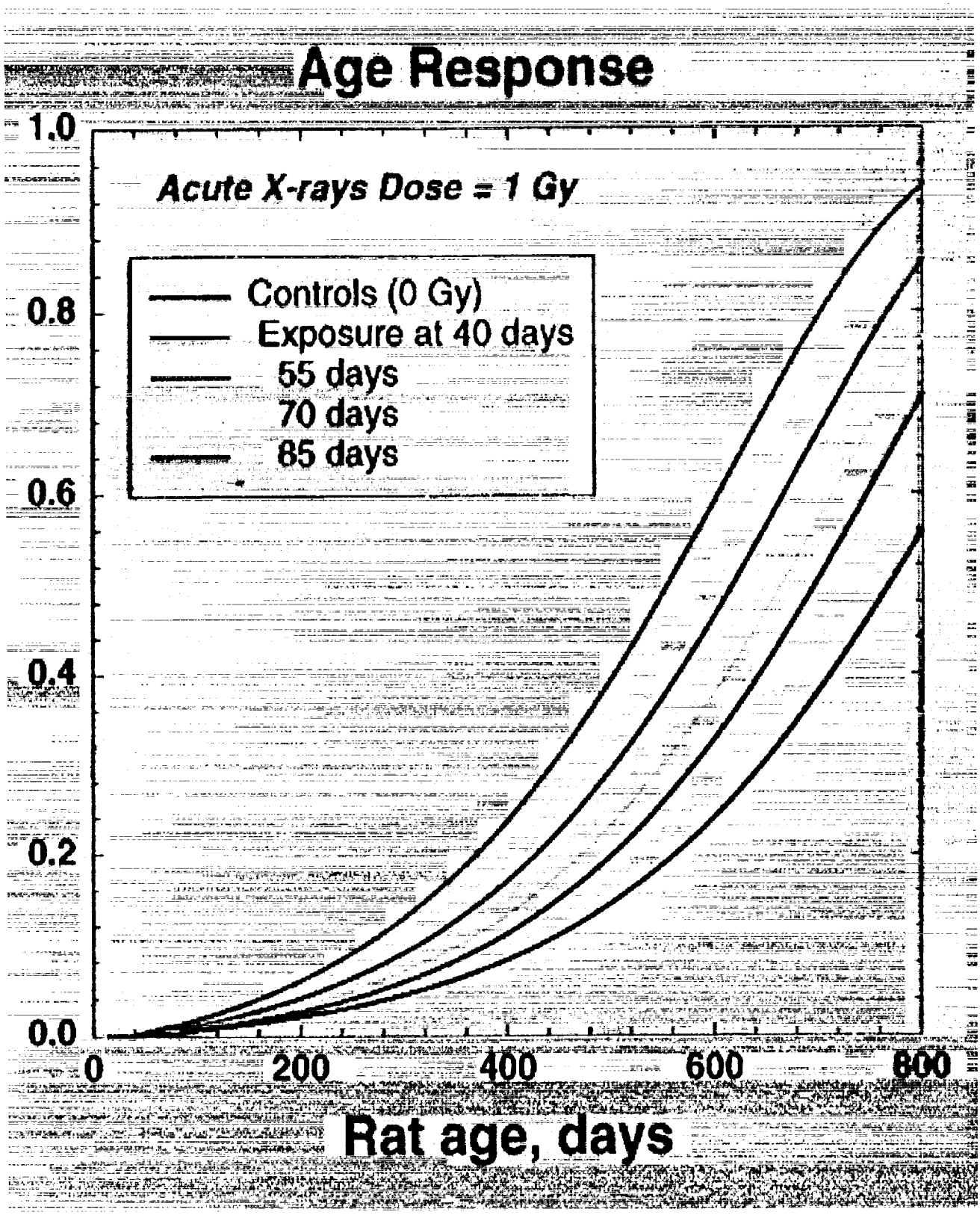


Fig. 25: An estimation of the change in the incidence rate as a function of age of the rats at the time of exposure of 1 Gy of photons.

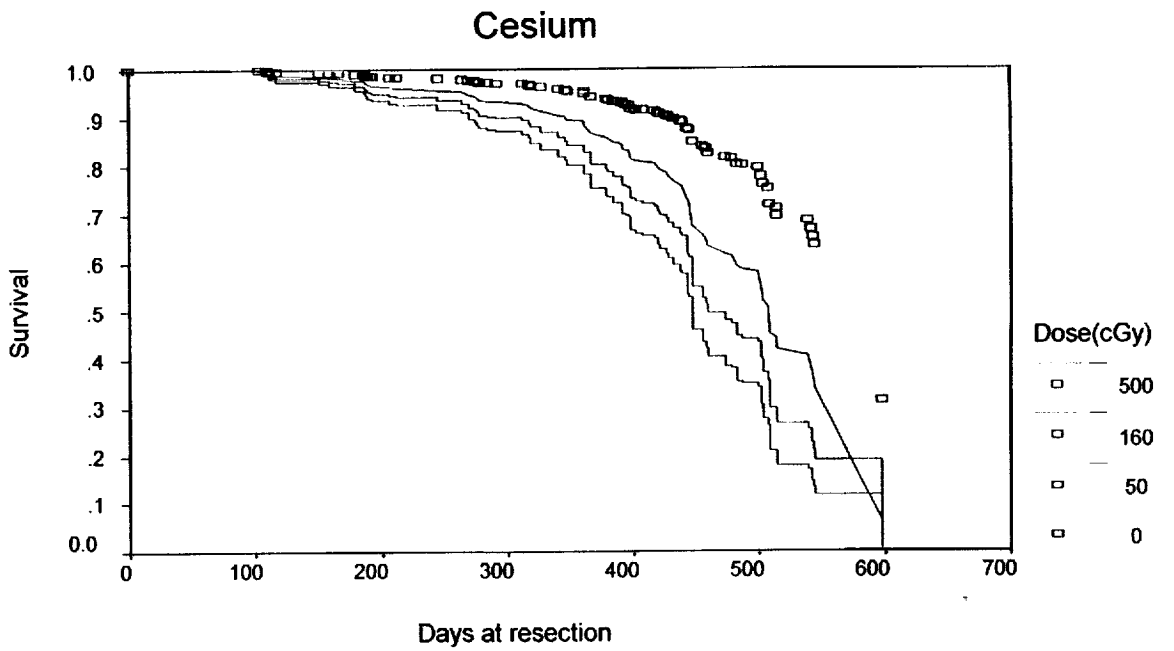


Fig. 26a. Preliminary results of a maximum likelihood analysis for relative risk for tumor-free survival as a function of time after irradiations with cesium-137 gamma rays. The cesium groups formed the baseline.

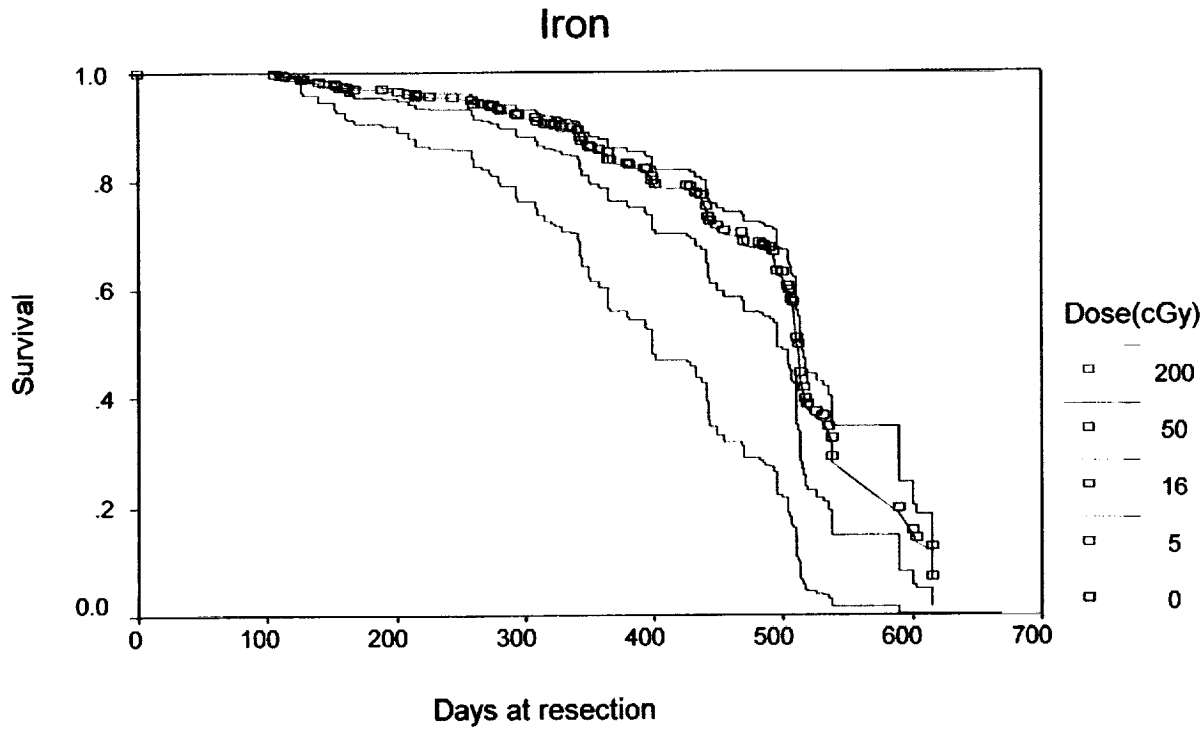


Fig. 26b. Preliminary results of a maximum likelihood analysis for relative risk for tumor-free survival as a function of time after irradiations with iron ions.

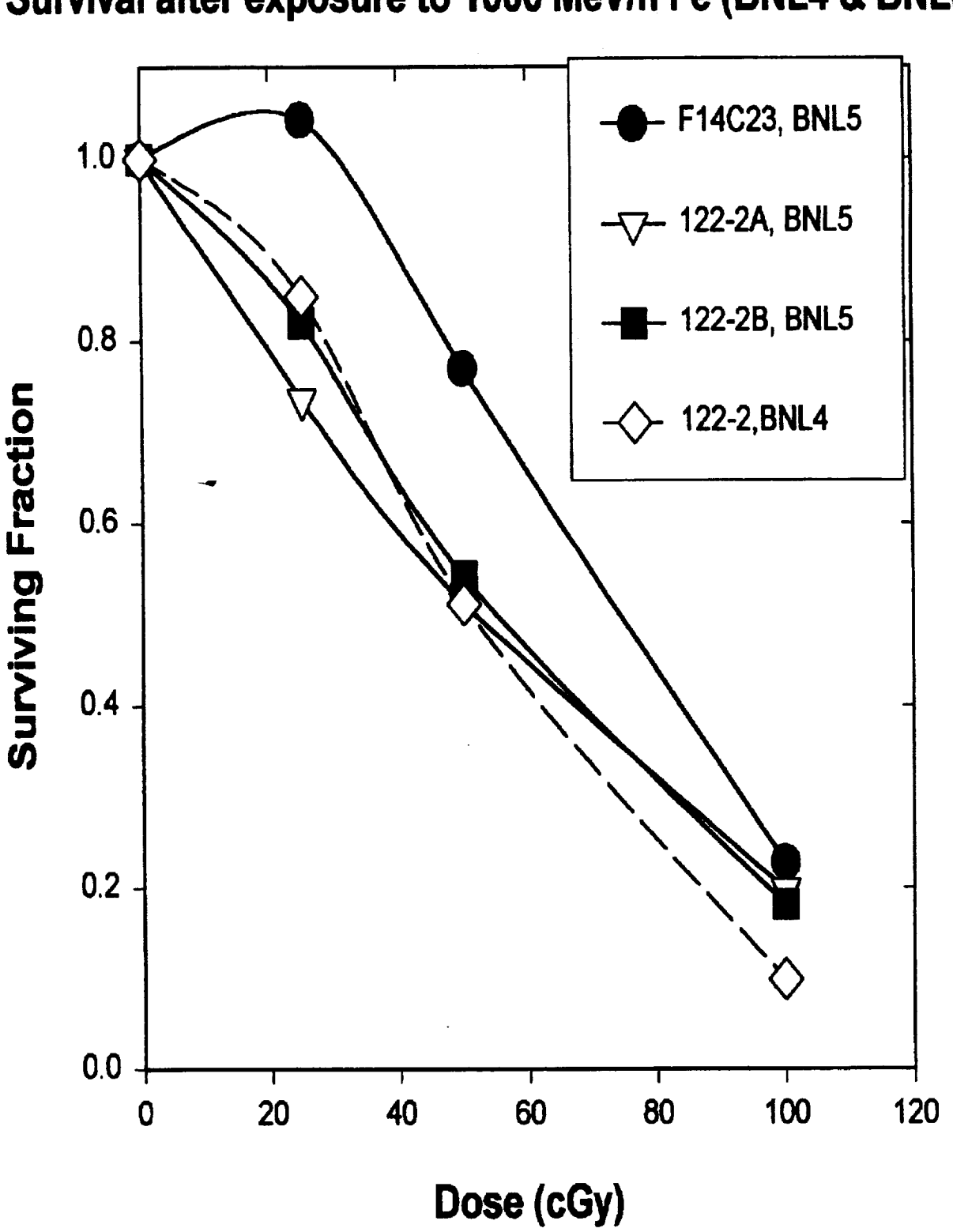


Fig. 27: Survival following exposure to 1000 KeV/n Fe particles.

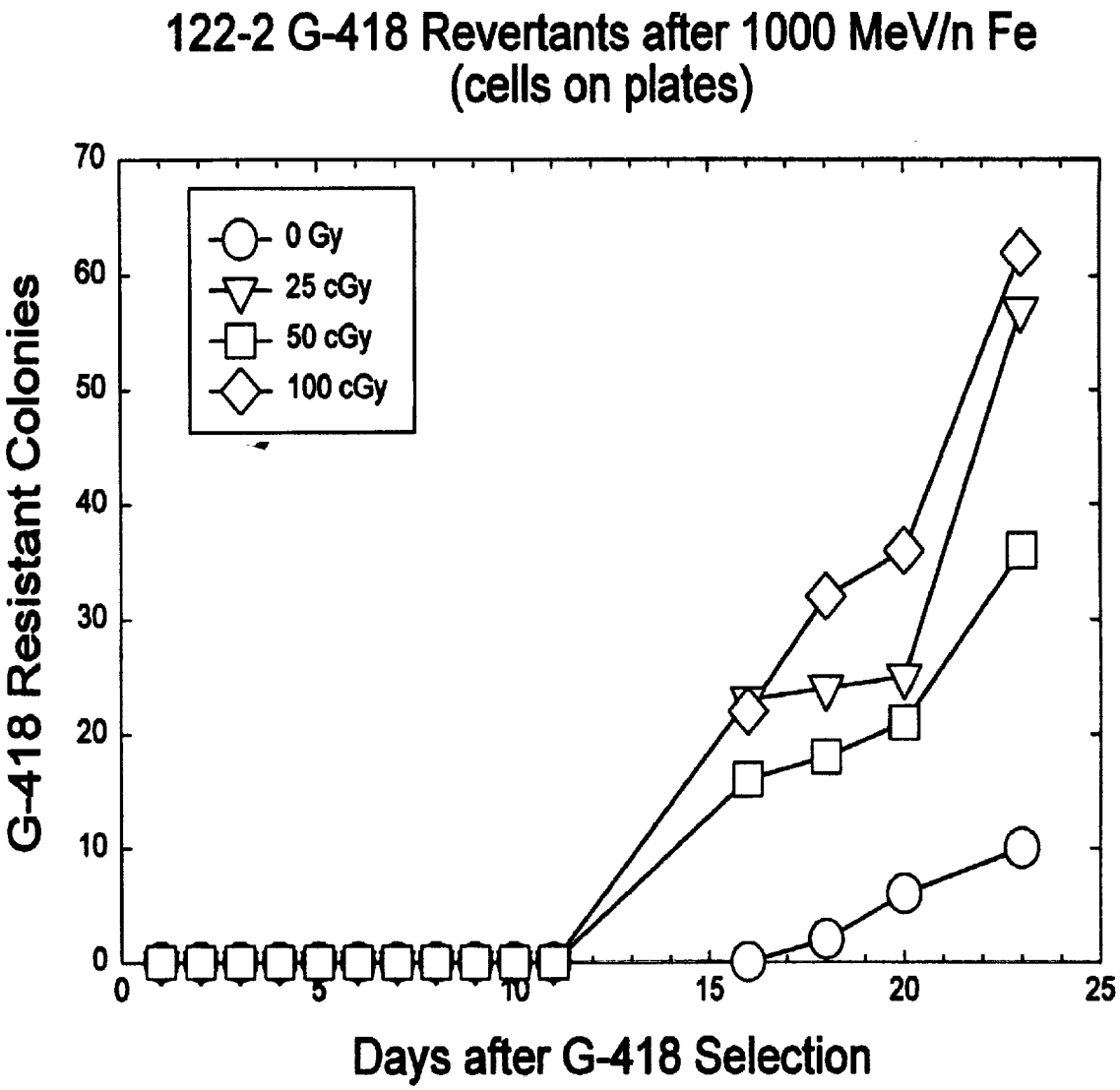


Figure 28: G-418 resistant revertants following 1000 MeV/n Fe.

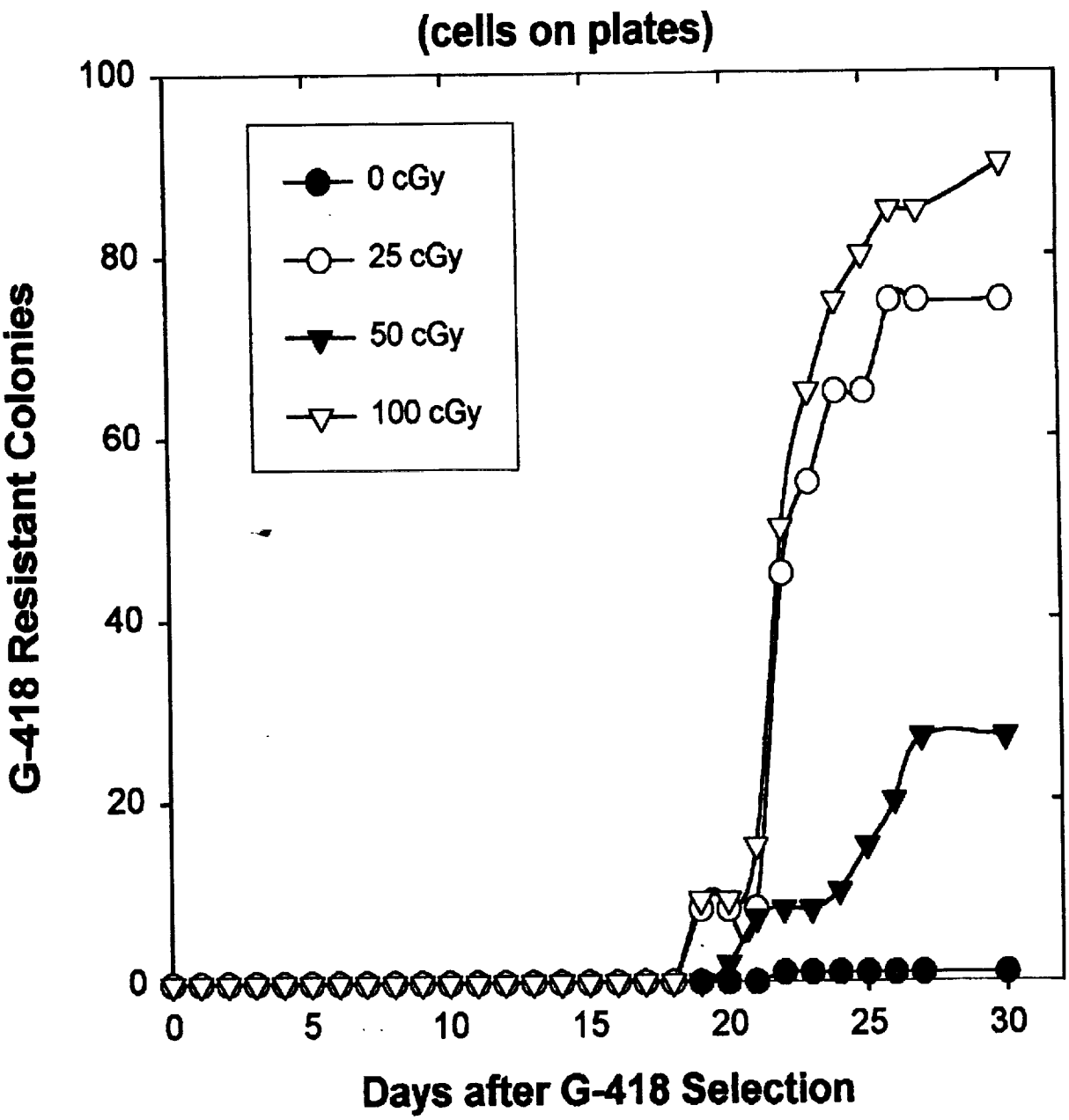


Figure 29: G-418 revertant cells; selection after first passage.

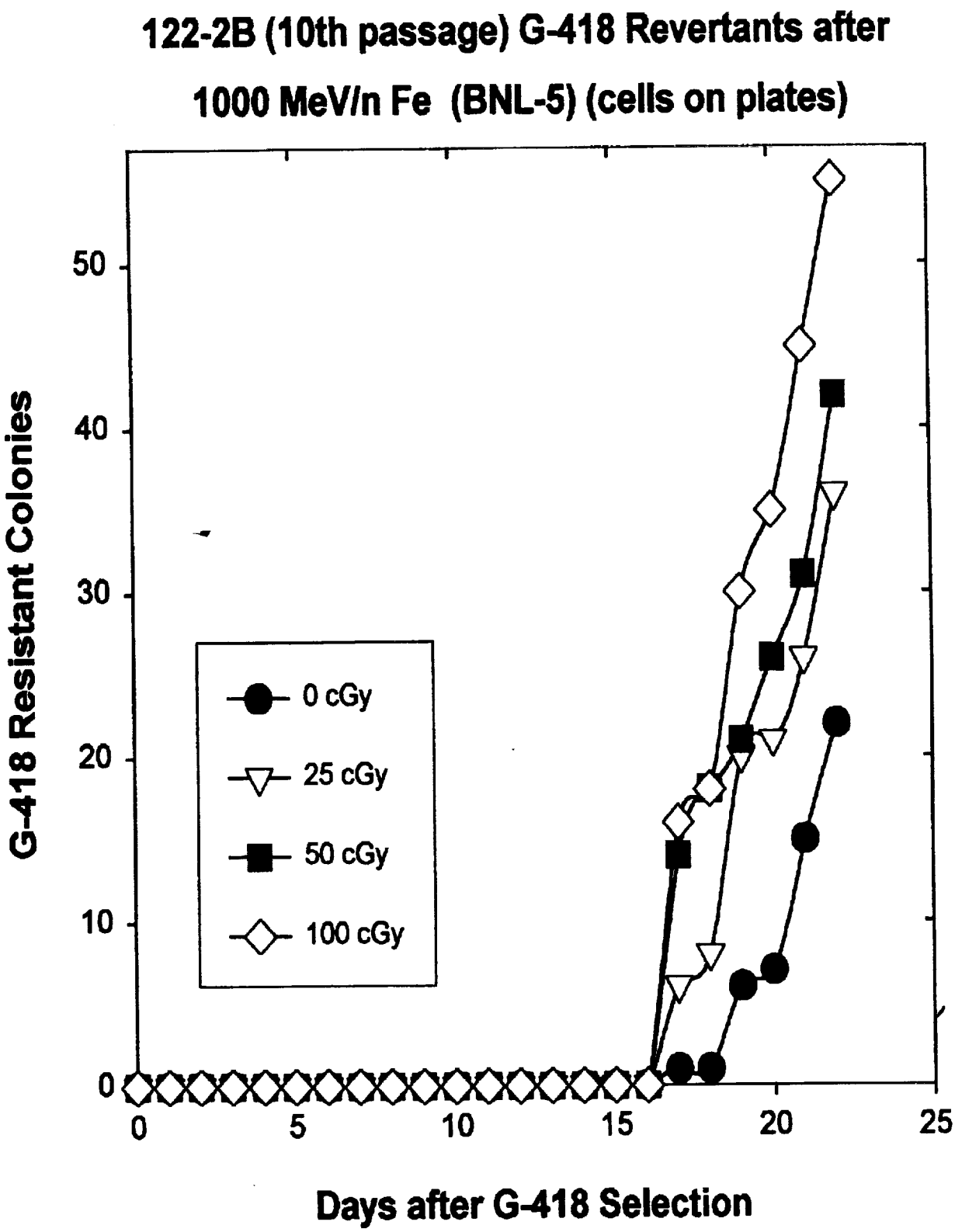


Figure 30: G-418 revertants after 10th passage of growth following exposure to 1000 MeV/n Fe.

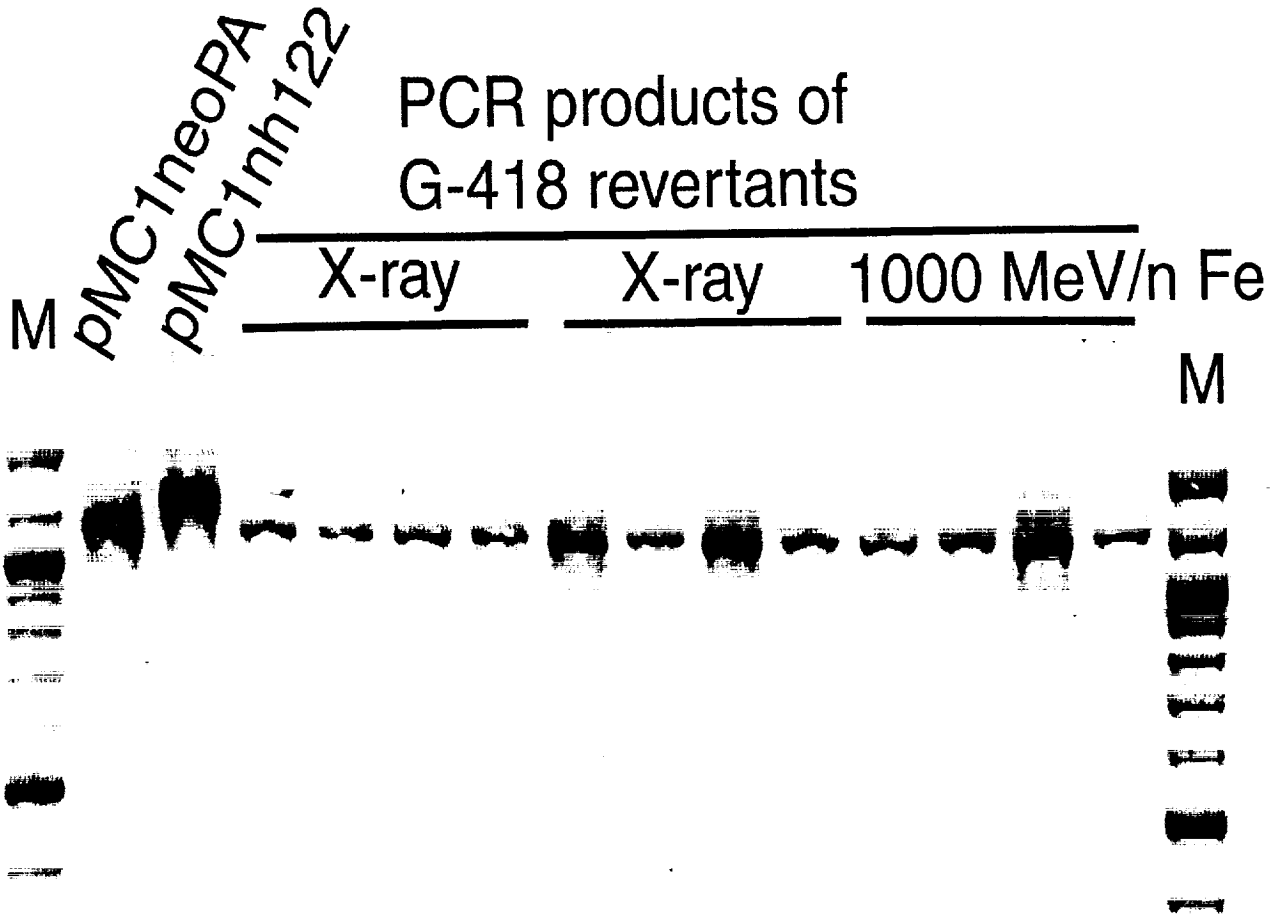


Figure 31: PCR analysis of deletion of the 122 bp insert in the *neo* gene.

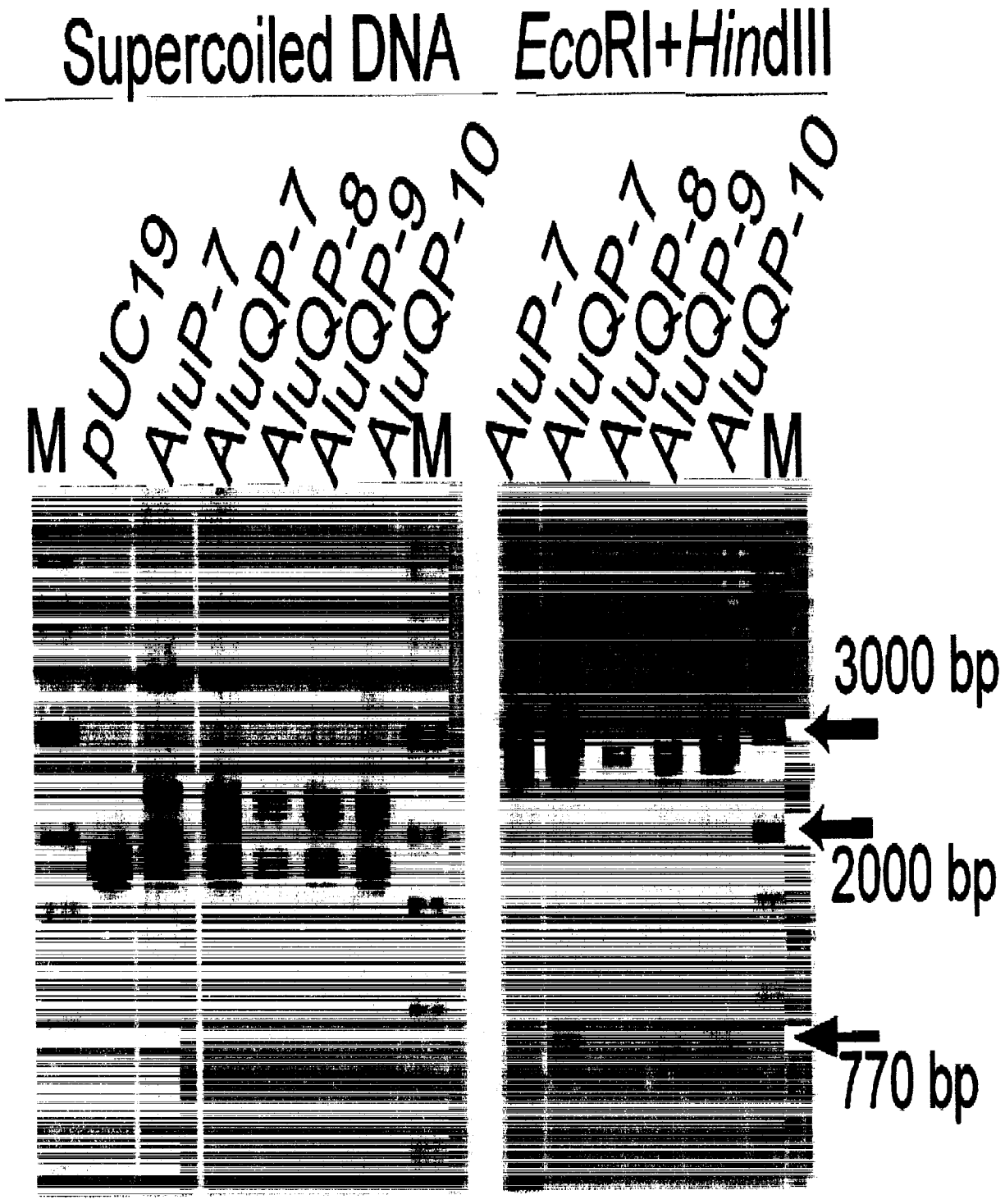


Figure 32: Analysis of Alu inverted repeats cloned into pUC19. Supercoiled DNAs (upper band in insert-containing plasmid while the lower band represents deletion products, uncut plasmids; EcoRI + HindIII, plasmid cut with those enzymes to generate the 770 bp inverted repeat. AluP-7, strain containing the perfect inverted repeat; AluQP-7-10, clones containing the inverted repeat with the 37 bp non palindromic center.

SIGNATURE SHEET
and
CONFIDENTIALITY STATEMENT

FINAL PROJECT REPORT

National Space Biomedical Research Institute

Radiation Effects Team

Project Title. **RADIATION EFFECTS CORE PROJECT**

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The information presented in this report is preliminary, proprietary, and confidential.

Signature_____

COVER PAGES

National Space Biomedical Research Institute

Radiation Effects Team

Project Title: **RADIATION EFFECTS: CORE PROJECT**

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In addition to the previous investigators, we have developed collaborations with other researchers with unique biological systems which are particularly valuable in our primary study. These potential collaborators include Dr. Marcelo Vazquez, Brookhaven National Laboratory, who has been working with us at the Brookhaven National Laboratory and; Drs.Gregory Nelson, and James Slater, who have been working with us at the NASA research center of Loma Linda University Medical Center.

EXECUTIVE SUMMARY

The risks to personnel in space from the naturally occurring radiations are generally considered to be one of the three or four most serious limitations to human space missions, as noted in two reports of the National Research Council/National Academy of Sciences (1996, 1998) and the NASA Critical Path Roadmap. The main objective of the Core Project of the Radiation Effects Team for the National Space Biomedical Research Institute is to study the consequences of radiations in space in order to develop countermeasures, both physical and pharmaceutical, to reduce the risks of cancer and other diseases associated with such exposures.

During interplanetary missions, personnel in space will be exposed to galactic cosmic rays, including high-energy protons and energetic ions with atomic masses of iron or higher. In addition, solar events will produce radiation fields of high intensity for short but irregular durations. The level of intensity of these radiations is considerably higher than that on Earth's surface, and the biological risks to astronauts is consequently increased, including increased risks of carcinogenesis and other diseases. Carcinogenesis from space radiation is one of the major health concerns for long term space travel. Protons are the most abundant component of the space radiation in both solar particle events and in the galactic cosmic rays. The corresponding dose-rates for proton exposure range from 10^{-3} to 0.1 Gy/hr, with possible accumulated doses of roughly 0.5–2 Gy in a large solar particle event or for interplanetary travel. These dose-rates are lower than those used in therapeutic treatment of cancers or encountered in epidemiological studies of A-bomb survivors.

This group is examining the risk of cancers resulting from low-dose, low-dose rate exposures of model systems to photons, protons, and iron by using ground-based accelerators which are capable of producing beams of protons, iron, and other heavy ions at energies comparable to those encountered in space. The specific aims of this work include *in-vivo* studies of carcinogenesis resulting from exposures to low doses of energetic heavy-ions, protons, and photons. We have successfully conducted a series of experiments using a 1-GeV iron beam at the Brookhaven National Laboratory and 250-MeV protons at Loma Linda University Medical Center's proton synchrotron facility. As part of these studies, this group is investigating the potential for the pharmaceutical, Tamoxifen, to reduce the risk of breast cancer in astronauts exposed to the level of doses and particle types expected in space. These data are essential for an improved evaluation of the cancer risks from radiation in space. Nevertheless, this is only the second large-scale study of this type and only the first including a study of a chemopreventive agent. Although the experiments are only in the preliminary stages, extensive data have been forthcoming and are reviewed in this report.

Theoretical studies are being carried out as part of this project in a collaboration between scientists at NASA's Johnson Space Center and Johns Hopkins University. The theoretical studies, in coordination with the experimental program, have provided methods and predictions which are being used to improve our evaluation of radiation risks to be encountered and to evaluate appropriate strategies for countermeasures. Continued collection and analysis of data from this project over the next three years will enhance the precision of our estimates of biologic response and reduce the unacceptable uncertainties associated with present risk assessments of activities in space which increase vulnerability and costs.

Although the work in this project is primarily directed toward problems associated with space travel, the problem of protracted exposures to low-levels of radiation is one of national interest in our energy and defense programs, and the results may suggest new paradigms for addressing such risks.

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I. PROJECT RESEARCH ACTIVITY

A. ORGANIZATIONAL STRUCTURE OF CORE PROJECT

The present Core Project evolved from the Phase-I and Phase-II NSBRI proposals to NASA and the original missions of the NSBRI. Our primary objective in the Phase-II proposal was to initiate new large-scale animal experiments to better determine the risks of cancers associated with space exploration, especially those associated with long-term interplanetary human missions. Until this series of experiments, there had been only one animal study to investigate the effects of ions of high atomic number and high energy, HZEs. That experiment was conducted by Alpen et al. (1993) with the Berkeley Bevalac, which has been out of commission for almost a decade. It provided data on carcinogenesis in the harderian gland of a mouse model as a function of linear energy transfer (LET) and has been a cornerstone for risk assessments in space during the intervening time. No such series of experiments had been conducted to evaluate whether drugs could be used to reduce the risk of cancer.

After extensive discussions and meetings during the developmental period of the NSBRI, we chose as our animal model the female Sprague-Dawley rat for reasons which we felt would improve the accuracy and the applicability of the results and allow experimental time lines that were consistent with the goals of NASA (go or no go by 2004) and the NSBRI (five-year funding):

1. It is one of the best characterized animal models for chemical and physical agents,
2. It has one of the best data bases for responses to photon radiations,
3. There were some data available on cancer induction in the Sprague-Dawley rat using neutrons, which would be useful in our estimating the dose ranges for the most effective experiments with HZEs,
4. It has been used successfully to establish the efficacy of one of the most widely used drugs in therapy, Tamoxifen, although at only higher doses to prevent recurrences rather than as a chemopreventor,
5. Human trials with Tamoxifen were underway at that time,
6. Tamoxifen is effective in the promotion and progression stages of cancer which could be a major advantage for exposures occuring in space,
7. Female Spraque-Dawleys have a high natural incidence of carcinomas of the breast, and high natural incidence generally considered to be a prerequisite for sensitivity to low doses of radiation. That is, statistically meaningful changes in a reasonable number of animals generally require a significant natural rate of occurrence,
8. In-vivo results in a mouse model already existed, so this would provide data for another species,
9. Rat experiments in space have a much better track record for success that mouse experiments, so the results of the ground experiments could be applied directly to ones in space with a greater likelihood for success,
10. The Sprague-Dawley was to be used extensively by other teams within the NSBRI, allowing direct intercomparisons of results,
11. The female breast is one of the most radiation sensitive tissues in humans, and breast cancer ranks as one of the most prevalent cancers and a major cause of death in women,
12. It was strongly recommended as the model of choice by the NSBRI External Advisory Council.

Animal experiments of this type were generally not done previously, presumably for good reasons. Such experiments are expensive. The opportunity for preliminary experiments to establish appropriate dose ranges and approximate levels of response, needed to establish number of animals to achieve a given accuracy, was essentially nonexistent. Only three facilities in the world, one at Brookhaven National Laboratory in New York State, one in Germany, and one in Japan, produced the necessary accelerator HZE beams. The costs for HZE beam time is millions of dollars a year, far in excess of any funds available from the NSBRI, and only about 150 hours a year are available for all space-biology irradiations in the U.S.A. Finally, no one had ever carried out such experiments at multiple facilities, including the Loma Linda University proton facility. The logistics of transporting thousands of animals between multiple facilities in isolated environments and keeping them alive subsequently for three or more years was at best a challenge.

It is to the credit of NSBRI and the research group that was organized that the logistics were established and appropriate transportation and irradiation equipment was designed and built. Because of our choice of animal model we were able to theoretically estimate the dose ranges with sufficient accuracy that even our first set of experiments were in the general ranges we expected. (This was despite our estimations of RBEs being considerably lower than those frequently proposed by some members of the scientific community.) The net result is a highly productive three years of research by what is now an outstanding team of researchers, which most certainly represent a unique national resource at this point. The success of the present project and team at costs comparable to that of teams in other areas of the NSBRI suggests that the limitations restricting such experiments in the past may no longer be applicable, and we should move forward to completion with this area of research.

Recognizing that this was potentially both a unique opportunity that might not be repeated and an expensive one at the same time, we encouraged our colleagues in other projects to join forces with us to maximize the productivity and usefulness of the irradiated animals and to provide us with data that we would ultimately need to extrapolate our results to humans in the space environment. Of the three other projects that successfully survived the review process, two had proposed to use the Sprague-Dawley, one to study Tamoxifen (Howard/Huso) and the other to look at cytogenetic aberrations to predict cancer risks (Williams). In both cases, we designed and built the irradiation devices and carried out the irradiations at the different facilities, although we required at least one individual from each project to participate. The third project (Sinden) was to use low-energy beams of low atomic number to study repeated DNA sequences using techniques that were established only for mouse models. In the latter case, although they carried out the research themselves, we provided guidance and even provided NSBRI funds from our Core Project, so they could hire a technician during the second year of the grant. At this point, the individuals within the Team work so well together, the project boundaries have become barely perceptible.

The final research structure that was established for the Core Project, then, is presented in Figure 1. The details of the individual projects in the Core will be discussed subsequently. However, the overall philosophy is that the Core Project provides the *in-vivo* results for risks of cancer and other diseases; the Chemoprevention Project provides the data for risk reduction with Tamoxifen; the Cytogenetics Project gives us data for chromosome aberrations for cells irradiated in the animal or in-vitro to provide us some mechanistic benchmarks; finally, the Core Project assembles all of the data to calculate risks in the animal model and to extrapolate to risks of humans in space.

Original Research Structure for the Radiation Effects Core Project

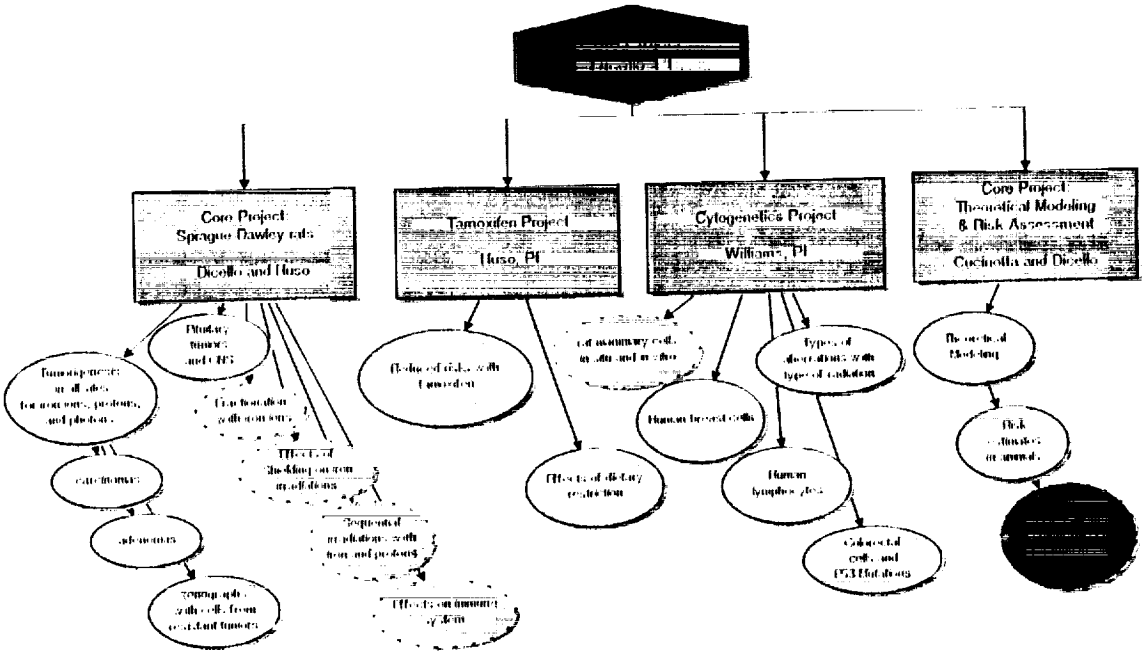


Fig. 1: Research structure for the Radiation Effects Core. Boxes enclosed in dashed lines represent preliminary or feasibility studies.

Within the next couple of years, the Booster Application Facility (BAF) at the Brookhaven National Laboratory will become operational. This facility is being funded by NASA and has been designed with the intention that it be used for biology experiments relevant to the space program. Presently, our NSBRI research represents the largest single user of beam time for space biology. There will be a significant increase in beam-time available for space biomedical research. In anticipation of this new era, we have tried to do as many preliminary or feasibility experiments as possible to offer some data for potential researchers designing future experiments. All of these experiments were included in our original proposal and peer reviewed, but were eliminated or reduced because of reduced funding. These include a small study to determine the effects of fractionation on the RBEs, the effects of HZEs or protons on the pituitary and CNS, the effects of increased shielding on the RBEs, and potential synergistic effects from combined irradiations of protons and HZEs. In space there is a continuous background of protons contributing the largest fraction of physical dose of any primary particle. Moreover, a significant fraction of that dose is from high-LET secondaries. Ground-based acute exposures to one particle type may not be an adequate simulation of the space environment (Dicello, 1992).

B. HYPOTHESES, OBJECTIVES & SPECIFIC AIMS FROM ORIGINAL PROPOSAL

The **underlying hypothesis** driving the original project was that there are innovative countermeasures, including novel spacecraft design and chemopreventive agents, which can be implemented to reduce radiation exposures in space and/or the subsequent biological consequences to personnel. We believed that the results of a study of cancers of the breast, lung, brain and leukemia and other observable diseases in rats and mice irradiated with energetic heavy ions (HZEs), protons, and photon could be used to establish risks in humans and to test the efficacy of potential countermeasures. As mentioned previously, until this program, only one large-scale study established the risk of carcinogenesis in an *in-vivo* study at lower doses of HZEs, the study of Alpen et al. (1993) that determined the risk of cancer in the harderian gland of mice. With the funding limitation, there existed a certain pessimism on the part of the scientific community that any additional study could be carried to completion in a timely fashion, particularly if it were to include an attempt to test the hypothesis that modest doses of drugs could reduce the risk of cancer at lower doses.

To establish that relevant experiments can be carried out effectively, we chose one animal model, the Sprague-Dawley rat, which has been used successfully for a variety of drug studies for which the data have likewise been successfully extrapolated to humans. Our specific hypotheses were:

Hypothesis I: *Studies of all types of tumor induction and other diseases in Sprague-Dawley rats and other animal models from low-dose irradiations with protons and HZEs will provide a basis for evaluating the relative carcinogenic risks from such particles.*
Hypothesis II: *Radiation damage leading to carcinogenesis may be mitigated with specific drugs implemented at reasonable doses during the promotion or progression stages rather than prior to exposure or during initiation.*

The **overall objective** of this Strategy was to find **safe, effective chemoprevention agents** by first determining the effectiveness of the radiations for producing cancers in relevant animal models in major tissues at risk and then testing the effectiveness of specific pharmaceuticals to reduce that risk **through intervention during promotion and progression** rather than during initiation.

Our specific aims for this project are:
Specific Aim 1: To design protocols for exposing animal models to accelerator-based high-energy proton and heavy-ion (HZE) beams, with detailed biostatistics for the numbers of animals exposed and the expected level of certainties.
Status: COMPLETE. Protocols for animal irradiations were developed and implemented during conduct of the study. The necessary number and delineation of animals into radiation quality and dose groups was determined in consultation with our biostatistican prior to each experiment.
Specific Aim 2: To design experimental procedures and to build the equipment and apparatus necessary to carry out the animal irradiations. To do the dosimetry and physics to characterize the irradiations.
Status: COMPLETE. Experimental procedures for animal irradiation were developed in collaboration with veterinarians and user facility liaisons. The animal exposures were conducted using custom-designed animal restrainers fabricated and tested at JHU.
Specific Aim 3: To coordinate irradiation of weanling female Sprague-Dawley rats at Brookhaven Laboratory for iron ions and Loma Linda for protons.
Status: COMPLETE. The Core Project coordinated the exposure of all irradiated rats and cell samples used by the Radiation Effects Team Project.

Specific Aim 4: To support a related project evaluating tumor formation in rat mammary glands and other sites with/without subsequent chemoprevention with Tamoxifen (TAM) in irradiation of animals. An underlying assumption is that funding will be available to accumulate data from the exposed animals for two years beyond the three-year period.
Status: COMPLETE. The Core Project has provided financial and analytical support to the Chemoprevention Project. The collaborative environment cultivated by the close working relationship of the Core and Chemoprevention Projects has lead to joint presentations of scientific data.
Specific Aim 5: To irradiate sequentially the same animals with both energetic protons and iron (in as short a possible time interval between irradiations) at dose ratios bracketing those in space and evaluate the level of synergistic, i.e., non-additive non-linear, effects of sequential or simultaneous irradiation.
Status: NOT FINANCIALLY SUPPORTED BY NSBRI. In response to recommendations by External Advisory Committee members and budgetary constraints, this specific aim was not funded by the NSBRI. However, in the interest of examining the feasibility of the proposed study, the PI employed his own internal funds to initiate a pilot study into the effects of sequential iron and proton irradiations.
Specific Aim 6: To model the biological results, so the data may be extrapolated to humans for the scenarios expected in space.
Status: CONTINUING. Biologic data is prepared and archived as it becomes available. Analyses of the data is periodically updated and investigated for trends and indications of new study directions. Over 65% of the exposed animals remain alive and under study. Preliminary results have been regularly provided to the NSBRI for inclusion in internal reports and conference proceedings.
Specific Aim 7: To establish risks relative to low-LET radiation.
Status: CONTINUING. Although only the initial cohort has progressed enough to merit detailed study, it appears an RBE for highly energetic iron ions is between 5 and 10, dependent upon the level of effect. Statistical uncertainty continues to mask low dose effects although progression of the more recent cohorts will considerably improve statistical precision.
Specific Aim 8: To establish safe pharmaceutical countermeasures to those risks.
Status: CONTINUING. Nearly 90% of the animals enrolled in cohorts associated with Tamoxifen treatment are still alive and under study. Initial indications suggest Tamoxifen is effective in reducing at least the short-term carcinogenic effects of iron ion, protons and photons. Follow-up of the study cohorts will be necessary to fully characterize the effects of Tamoxifen administration.

We considered the studies in this three-year project to be the foundation for a broader effort to address efficiently the unresolved issues associated with interplanetary human missions, as discussed in the original proposal and the Research Strategy for the Radiation Effects Program. Our long-term aims beyond the scope of this project or the three year Radiation-Effects Program are:

a. to examine tumorigenesis in the Sprague-Dawley rat with at least two other particles between protons and ions;
b. to examine tumorigenesis as a function of shielding thickness;
c. to examine tumorigenesis in an appropriate mouse model and a large-animal model at one low-dose equivalent to the proton dose expected in space;
d. to irradiate the rat model sequentially with both energetic protons and iron, to evaluate the level of synergistic, i.e., non-additive, effects, and more closely approximating the space environment;
e. to evaluate the potential of other possible chemopreventors such as Dehydroepiendrosterone (DHEA), Sulindac, or Retinoids; and
f. to perform subsequent examinations of the animals irradiated in the original three-year period and held long-term.

C. STUDY DESIGN AND PROCEDURES

The SD mammary gland has been extensively detailed physiologically (e.g., Russo and Russo, 1990) and has been the subject of numerous photon (Welsch et al., 1981; Bond et al., 1960; Shellabarger et al, 1966) and neutron experiments (Shellabarger et al., 1980; Shellabarger et al., 1978) as well as a Tamoxifen study (Welsch et al., 1981). The ability to induce mammary carcinomas in SD rats is being studied for both iron ions and protons. Highly energetic iron ions have historically been considered the most relevant heavy-ion component of the galactic cosmic ray (GCR) environment. Iron ions are densely ionizing and are relatively abundant as compared with other GCR particles. It is clear that high Z high energy (HZE) particles, especially iron ions, are a significant contributor to the risk of carcinogenesis. Protons comprise approximately 87% of the GCR fluence between 100 MeV/amu and 10 GeV/amu and nearly all of the fluence and dose equivalent from solar particle events (NCRP, 1989). Protons are the most abundant ionizing particle in free space and contribute the greatest fraction of total absorbed dose (Dicello, 1992). The abundance of protons both as a primary ionizing radiation and secondary fragment makes this particle of great importance, particularly considering that perhaps half of the high LET dose from GCR can a direct result of the protons (Dicello, 1992).

The number of rats irradiated for the Core Project during the funding period are detailed by cohort in Table 1. Note that, because of the scarcity of data for the Spraque-Dawley rat irradiated with heavy ions, it was necessary to determine to optimal dose range, both with respect to tumor induction, but also with regard to other diseases and lethality, in order to optimize the experiments. Prior to each cohort irradiation, the status of the previously irradiated animals was evaluated to determine the appropriate size of each group in order to achieve the statistical precision desired. In addition to the animal listed in Table 1, the Core Project coordinated the irradiation of additional rats and cell lines as required by other Projects of the Radiation Effects Program.

Irradiated Female Sprague-Dawley Rats in Study

	May-98	May-99	Nov-99	Apr-00	TOTAL
Cesium (sham) @ BNL	36	20	40	0	96
Cesium (50 rad) @ BNL	36	20	40	0	96
Cesium (90 rad) @ BNL	0	20	0	0	20
Cesium (160 rad) @ BNL	35	20	40	0	95
Cesium (300 rad) @ BNL	0	20	20	0	40
Cesium (500 rad) @ BNL	36	20	20	0	76
Cesium (900 rad) @ BNL	0	20	0	0	20
Iron (sham) @ BNL	70	40	60	0	170
Iron (5 rad) @ BNL	70	40	60	0	170
Iron (9 rad) @ BNL	0	40	0	0	40
Iron (16 rad) @ BNL	71	40	60	0	171
Iron (30 rad) @ BNL	0	40	40	0	80
Iron (50 rad) @ BNL	69	40	25	0	134
Iron (90 rad) @ BNL	0	40	0	0	40
Iron (160 rad) @ BNL	0	40	0	0	40
Iron (200 rad) @ BNL	18	40	0	0	58
Iron 16 + Proton 160	0	11	0	0	11
Proton (sham) @ LLU	36	48	0	20	104
Proton (50 rad) @ LLU	36	56	0	50	142
Proton (90 rad) @ LLU	0	56	0	60	116
Proton (160 rad) @ LLU	36	48	0	30	114
Proton (300 rad) @ LLU	0	48	0	0	48
Proton (500 rad) @ LLU	36	48	0	0	84
Proton (900 rad) @ LLU	0	48	0	0	48
Cobalt (sham) @ LLU	18	20	0	20	58
Cobalt (50 rad) @ LLU	18	20	0	58	96
Cobalt (90 rad) @ LLU	0	20	0	60	80
Cobalt (160 rad) @ LLU	18	20	0	32	70
Cobalt (300 rad) @ LLU	0	20	0	0	20
Cobalt (500 rad) @ LLU	18	20	0	0	38
Cobalt (900 rad) @ LLU	0	20	0	0	20
Cesium (sham) @ JHU	18	20	0	0	38
Cesium (50 rad) @ JHU	18	20	0	0	38
Cesium (90 rad) @ JHU	0	20	0	0	20
Cesium (160 rad) @ JHU	18	20	0	0	38

Table 1. Different irradiation scenarios and the number of rats for each scenario.

Almost 3000 weanling female Sprague-Dawley rats were shipped directly to the irradiation facilities at Brookhaven National Laboratory (BNL), Loma Linda University Medical Center (LLUMC) or The Johns Hopkins University (JHU). The animals were irradiated at approximately 60 days of age within a Lucite holding apparatus designed and fabricated at JHU. The rats were positioned on their sides with their breasts perpendicular to the incident beam.

The first series of irradiations at Hopkins, BNL, and LLUMC were to obtain base-line data and rough RBEs. HZE irradiations with iron ions, 1 GeV initial mean energy, were carried out at the Alternating Gradient Synchrotron facility at BNL in May 1998, May 1999 and November 1999. The absorbed-dose levels at which the rats were exposed were approximately a factor of ten less than the corresponding proton and photon groups to compensate for the estimated increased effectiveness per unit dose of iron. Proton irradiations employed incident protons of 250 MeV and were conducted at the LLUMC proton synchrotron in July 1998, May 1999 and April 2000. Irradiation of rats with photons was carried out at the host facilities during each of the six beam runs at BNL and LLUMC to establish baseline radiosensitivity and relative biological effectiveness (RBE) factors. Photon irradiations were also conducted at JHU to assess possible effects of post-irradiation travel stresses upon biological outcome.

As noted in Table 1, approximately half of the 1999 and 2000 irradiated rats were administered Tamoxifen, a chemopreventive agent being studied in conjunction with Dr. David Huso, PI, as part of the Program of the Radiation-Effects Team. The administration of Tamoxifen to the animals was initiated approximately one month after irradiation. Initially, we intended to administer the drug for sixty days to compare with existing studies in the literature because our goal was to determine if the drug could reduce the risk rather than the most effective regimen. Upon recommendation by the External Advisory Committee, Tamoxifen has been administered continuously to the rats over the entire lifetime of the animals with a corresponding increase in cost.

Approximately three weeks after irradiation, the animals were transported to Johns Hopkins University for evaluation and care. The rats are housed two per cage in micro-isolator units under a 12 hour light, 12 hour dark regimen. All animals irradiated in 1998 and the Tamoxifen-treated 1999 and 2000 rats are provided food and water ad libitum. Because we found Tamoxifen treatment to result in lower body weight, based on initial base-line data, the non-Tamoxifen-treated rats of the 1999 and 2000 cohorts are under diet restriction to keep their weight similar to the Tamoxifen-treated groups.

Number of Rats Still Alive in each Category as of 9/13/00

	May-98	May-99	Nov-99	Apr-00	TOTAL
Cesium (sham) @ BNL	9	18	40	0	67
Cesium (50 rad) @ BNL	0	20	40	0	60
Cesium (90 rad) @ BNL	0	18	0	0	18
Cesium (160 rad) @ BNL	2	18	39	0	59
Cesium (300 rad) @ BNL	0	17	20	0	37
Cesium (500 rad) @ BNL	1	18	19	0	38
Cesium (900 rad) @ BNL	0	15	0	0	15
Iron (sham) @ BNL	7	36	59	0	102
Iron (5 rad) @ BNL	4	35	60	0	99
Iron (9 rad) @ BNL	0	34	0	0	34
Iron (16 rad) @ BNL	1	33	59	0	93
Iron (30 rad) @ BNL	0	37	40	0	77
Iron (50 rad) @ BNL	1	34	24	0	59
Iron (90 rad) @ BNL	0	33	0	0	33
Iron (160 rad) @ BNL	0	33	0	0	33
Iron (200 rad) @ BNL	0	0	0	0	0
Iron 16 + Proton 160	0	8	0	0	8
Proton (sham) @ LLU	5	45	0	19	69
Proton (50 rad) @ LLU	6	54	0	50	110
Proton (90 rad) @ LLU	0	53	0	60	113
Proton (160 rad) @ LLU	3	41	0	30	74
Proton (300 rad) @ LLU	0	39	0	0	39
Proton (500 rad) @ LLU	0	36	0	0	36
Proton (900 rad) @ LLU	0	11	0	0	11
Cobalt (sham) @ LLU	4	18	0	20	42
Cobalt (50 rad) @ LLU	2	20	0	57	79
Cobalt (90 rad) @ LLU	0	17	0	60	77
Cobalt (160 rad) @ LLU	3	19	0	32	54
Cobalt (300 rad) @ LLU	0	18	0	0	18
Cobalt (500 rad) @ LLU	1	16	0	0	17
Cobalt (900 rad) @ LLU	0	8	0	0	8
Cesium (sham) @ JHU	2	20	0	0	22
Cesium (50 rad) @ JHU	1	20	0	0	21
Cesium (90 rad) @ JHU	0	19	0	0	19
Cesium (160 rad) @ JHU	2	19	0	0	21

Table 2. Number of animal still alive as of Sept., 2000.

The rats are under daily surveillance and are periodically manually palpated for mammary nodules. Palpable nodules of diameter greater than 2 cm are excised and the animal is returned to the colony. All surgically excised or necropsied mammary nodules are histopathologically classified by veterinary pathologist in a blind evaluation, and the classification is confirmed blindly by a second veterinarian. Upon death, each animal undergoes a complete gross examination with representative samples from all major organs fixed and archived for further study.

D. RESULTS AND ANALYSIS

The number of animals alive as of September 13, 2000 is delineated in Table 2 by cohort and group. Over 65% of the entire inventory of study animals are still alive at the present time, meaning that a large amount of data is not yet available for analysis. The analyses provided in this report have been generated with less than complete data, are preliminary and confidential, and, unless otherwise noted, represent only the 1998 irradiated animals.

A preliminary study of the primary cause of death (Figure 2), as determined by gross examination, indicates that the animals are dying primarily from complications associated from pituitary-related diseases, not as a direct result of mammary tumors. This is in part because palpable mammary neoplasms are regularly resected prior to lethal damage up to the host. The high rate of death, particularly among the sham and low dose groups, is a related to the naturally high incidence of enlarged pituitaries as this strain of rat ages. When the primary cause of death is attributed to mammary tumors, this includes both deaths from the tumor and complications including surgery.

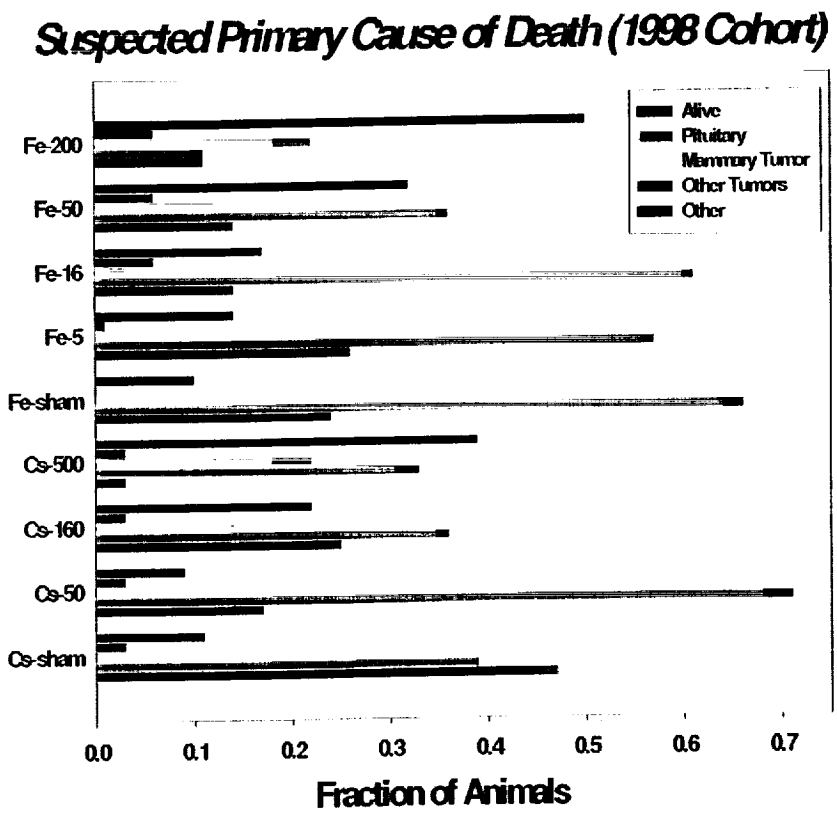


Fig. 2. Preliminary causes of death.

Figure 3 shows the number of mammary tumors resected, as a function of dose and radiation type. The number of tumors increases with increasing dose of a radiation type. The small count of Iron-200cGy tumors is a reflection of the reduced

number of animals in that group. Slightly less than half of all histopathologically classified mammary tumors are carcinomas. There does not appear to be a strong trend in the carcinoma/fibroadenoma ratio with dose or radiation type. It should be noted that each animal has 12 mammary glands, each of which can develop one or more tumors. Thus the same number of tumors in groups of the same size, such as shams and low dose groups, does not mean the same number of animals with tumors.

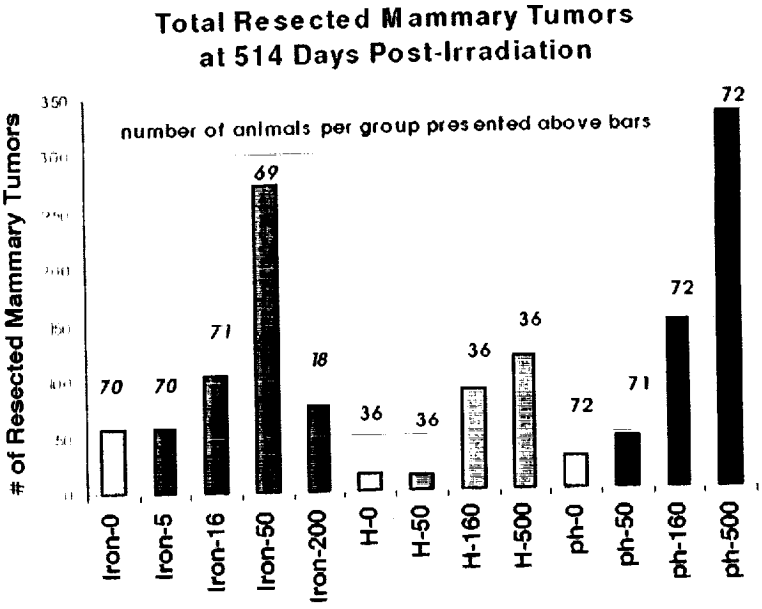


Fig. 3. The number of excised tumors observed as a function of radiation type and dose.

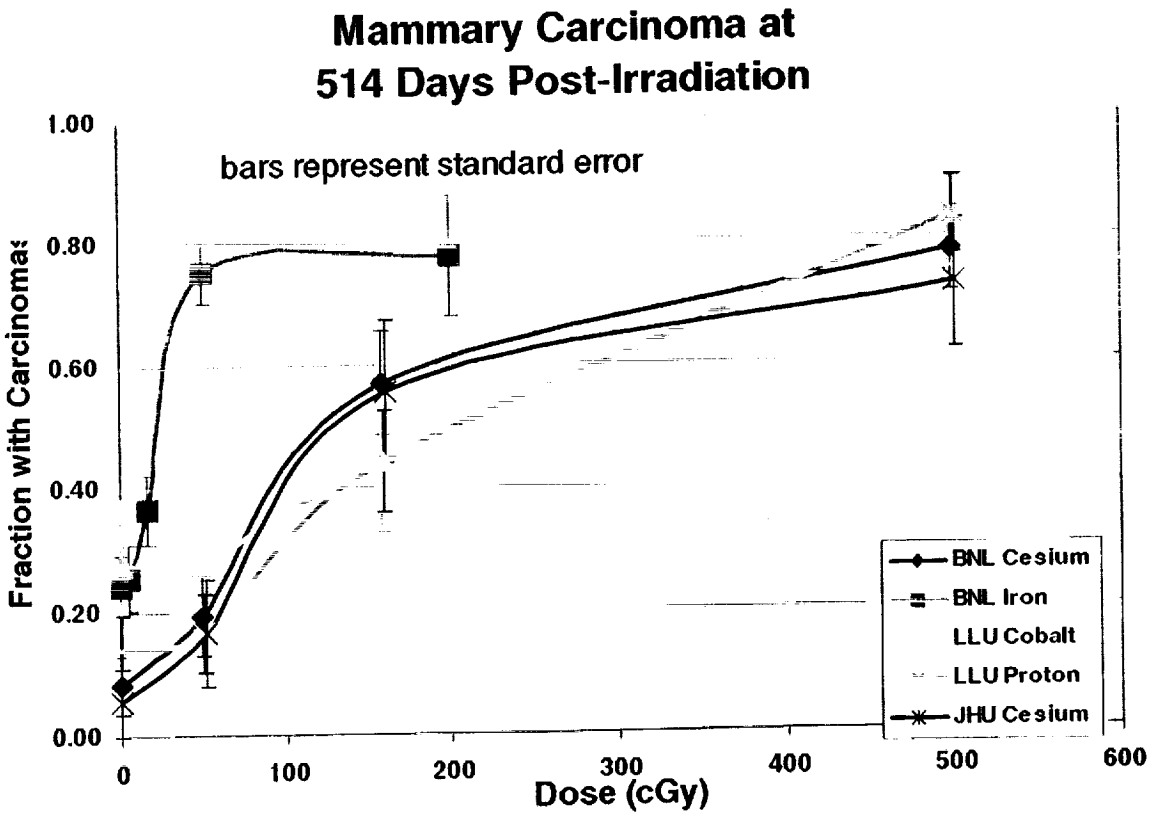


Fig. 4. Fraction of mammary carcinomas observed in each group at 514 days after irradiaton.

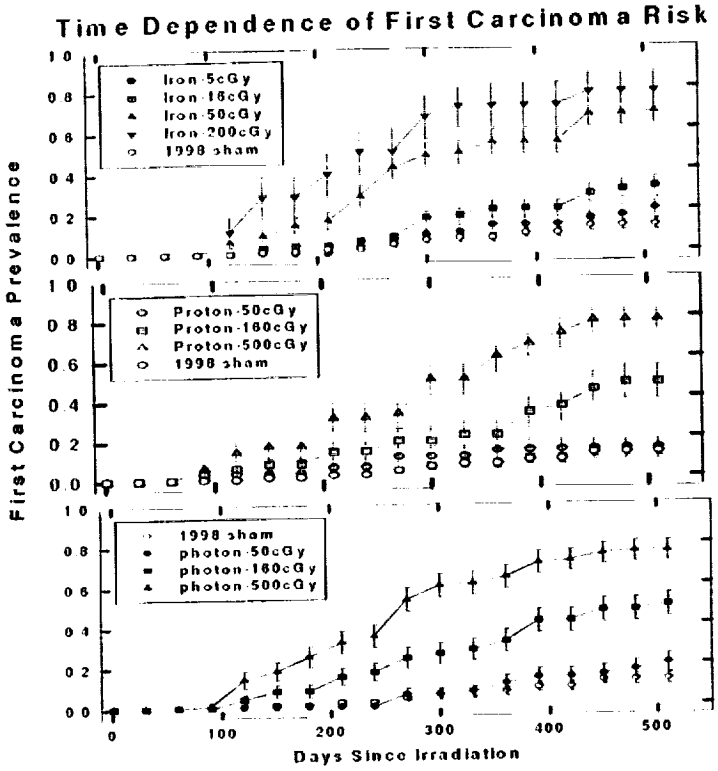


Fig. 5. Prevalence of first carcinoma as a function of time after irradiation.

The fraction of animals in each radiation dose/type group with a resected mammary carcinoma is shown in Fig. 4. A statistical analysis of proportions indicates there is no difference between each individual sham group; therefore, at this point, they are being pooled for subsequent analyses. The group of rats irradiated with Cesium at BNL, those irradiated with Cesium at JHU, and those irradiated with Cobalt at LLUMC were compared within each dose level and also found to be statistically similar and therefore pooled into single photon groups at each dose to increase statistical power. Analyses demonstrate that iron-5cGy ($p=0.14$) is not statistically different from the shams at this time. The proton-50cGy ($p=0.91$) or photon-50cGy ($p=0.53$) group responses are also not different from the shams at 514 days post-irradiation. The higher dose groups of each radiation type are significantly different ($p<0.05$) from the shams. Within the proton and photon irradiated animals, the response from each dose group is unique.

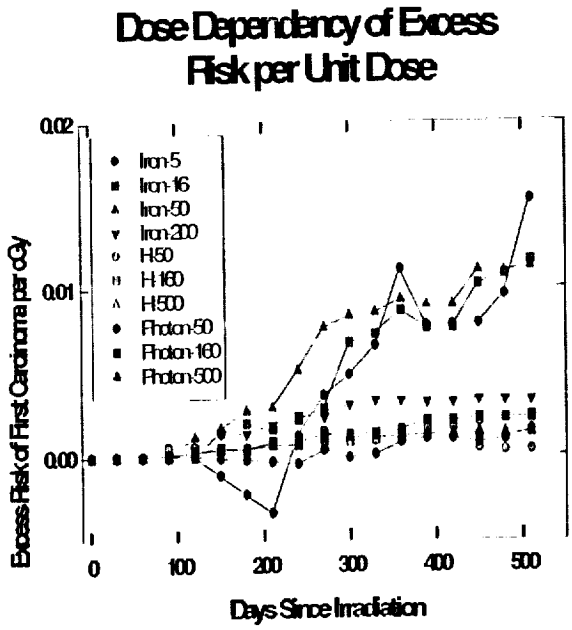


Fig. 6. Excess risk per unit dose.

Prevalence of first carcinoma as a function of time after irradiation is presented in Fig. 5 for each radiation group. The top graph illustrates first carcinoma response of iron-irradiated animals versus shams. A paired sample t-test was conducted and all curves for iron irradiation were significantly different from the shams except for iron-5cGy ($p=0.54$). Similar analyses were performed on the proton and photon curves within each radiation type. It was found that all curves differed significantly from

the sham curves, including the curves for the lowest doses of protons and photons. When comparing the proton curve versus the photon curve at the same dose, it was found that only the highest dose of 500cGy resulted in statistically similar curves. This may be significant in that it is frequently assumed in risk assessment that the carcinogenic effects of protons and photons are similar at identical doses.

To evaluate the linearity of effect per unit dose, excess risk of a first carcinoma per cGy was plotted for each dose group in Fig. 6. As expected, the effect per unit dose is greater for the iron ions than the protons or photons. The notable exception is the iron-200cGy group in which the excess risk is diminished. This effect is likely caused by the fact that, at this high dose, few animals remain without a carcinoma, so the pool of susceptible animals is diminished.

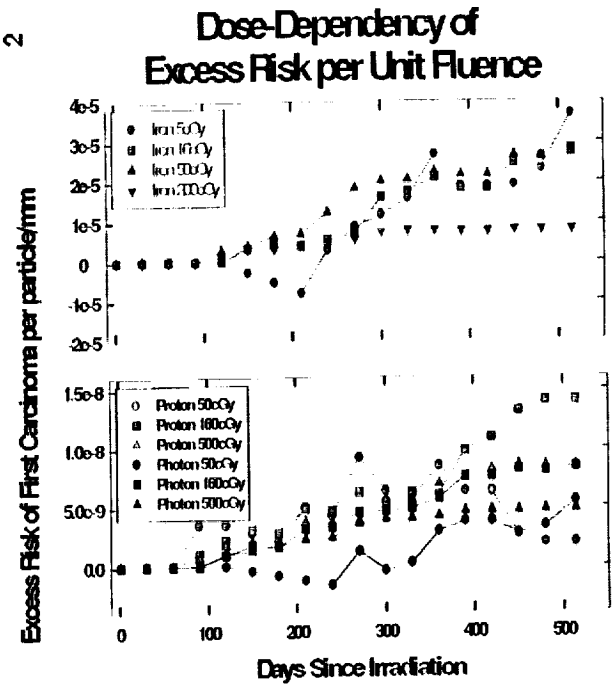


Fig. 7. The excess risk per unit particle fluence.

Again, because we did not know responses, we chose doses that would likely bracket the regions of interest. The tight grouping of the 5cGy, 16cGy and 50cGy iron groups is one indication that we are within the linear response region of the iron dose scale, at least up to 514 days post-irradiation. At 514 days, a RBEs between 1 and 10 appear consistent with the data, dependent upon the time and dose-region of interest.

The carcinogenic effectiveness per unit particle of iron, protons, and the particle equivalent for photons are shown in Fig. 7.

In an effort to correct our analyses for the number of animals at risk at any given time, a Kaplan-Meier survival analysis was performed (Fig. 8). A Mantel-Haenszel log rank test of KM survival estimators indicates no difference between shams and iron 5cGy ($p=0.07$). The lowest-dose proton and photon groups are also not significantly different from the sham response. A comparison

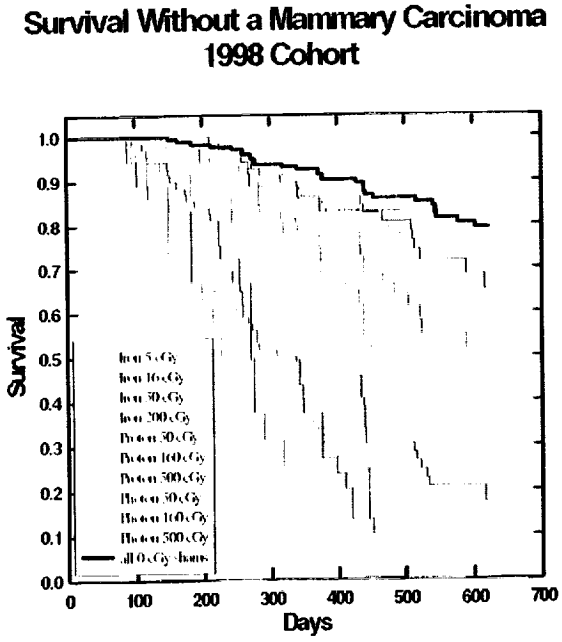


Fig. 8. Results of a Kaplan Meier survival analysis.

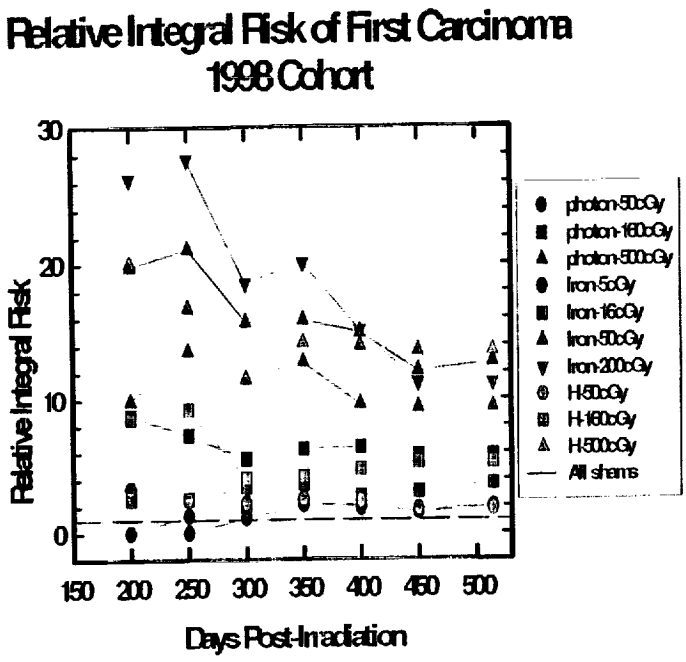


Fig. 9. Relative risk as a function of time since irradiation.

between proton and photon groups at the same dose level shows no significant difference.

The relative risk (RR) of a first carcinoma (in reference to the sham group) versus time post-irradiation is plotted in Fig. 9. The relative risks integrated with time demonstrate the strong dose dependence of time to first carcinoma. The fact that the RR is decreasing with time is an indication that the sham animals are developing mammary carcinomas at a greater relative rate that the highest dose groups. The near linearity of the moderate and low dose groups through time may be interpreted as support that the relative rate of cancer development is similar to that in the sham group. Such a study of the time-dependence of risk is not possible with most in-vivo animal studies as they typically utilize prevalence at the end of study as the major endpoint.

Figure 10 illustrates the effect of using an additional 11.5 cm lucite shield when irradiating the animals with iron ions. This amount of lucite reduces the average energy of the beam to about 600 MeV/amu, a typical beam energy used previously at the Berkeley Bevalac. Although the spectrum of radiation present behind the Lucite is of a lower energy and lower atomic mass and has a different lineal energy distribution, no measurable difference in carcinogenic effect is seen.

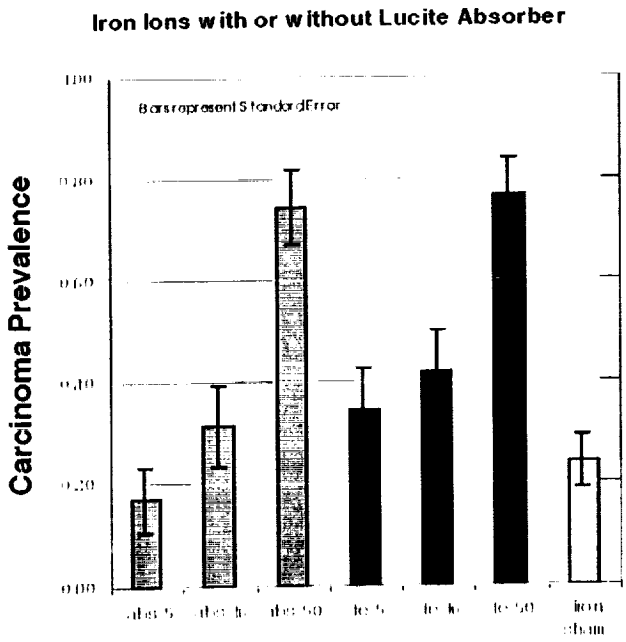


Fig. 10. A comparison of prevalence with lucite placed upstream of the rat during irradiation.

The animals treated with Tamoxifen as a chemopreventative agent are now approximately one year post-

irradiation. These results will be discussed in detail in the Final Report for the Chemoprevention Project, but the care of these animals and the analysis of the data are a major commitment for this project. For that reason, a brief summary is presented here. Figure 11 shows the carcinoma prevalence at 378 days post-irradiation. It is clear that Tamoxifen reduces the occurrence of mammary carcinomas at higher radiation doses. The Tamoxifen is less effective on the iron-irradiated animals. Interestingly, proton irradiated animals do not show as dramatic a reduction as photons, perhaps as a result of LET or track structure differences of the protons or their secondary radiations.

Current radiation risk assessment practices dictate that the total risk from a mixed radiation environment is simply the sum of the risks from each component. We are unaware of any in-vivo validation of this assumption as it relates to carcinogenesis. We initiated a small pilot study whereby 11 rats were irradiated with 16cGy of iron and then two days later, 160cGy of protons. Our findings,

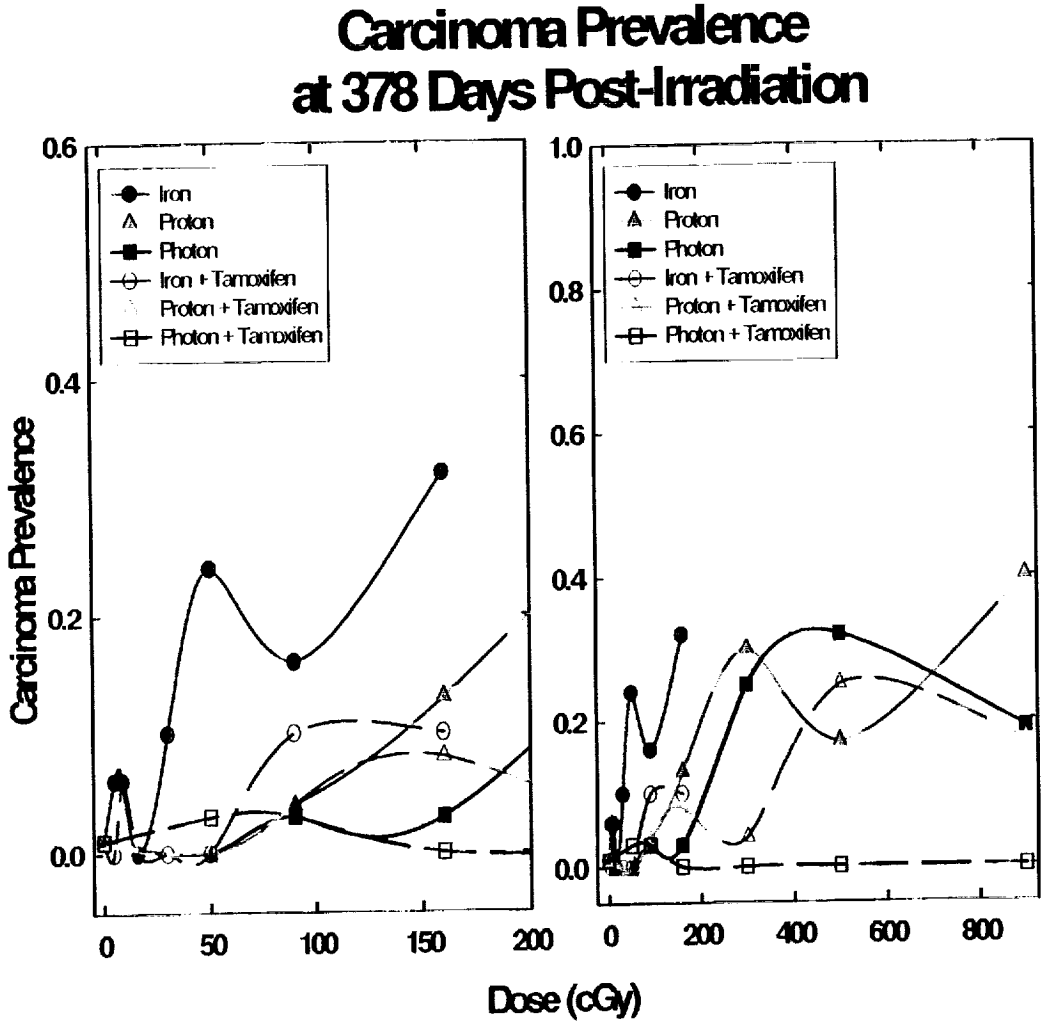


Fig. 11. Prevalence of cacinomas with and without subsequent Tamoxifen.

shown in Fig. 12, demonstrate that there may be a greater response than what would be expected by the summation of risk from each dose component. The risk for each component was determined by linear regression of the dose response curves from the iron and proton irradiated animals. Although this data involves a small sample size and unresected tumors as opposed to classified tumors as in previous studies, it suggests that the study of a mixed radiation environment merits further evaluation.

A small pilot study of the effect of fractionation of the iron dose was initiated more recently because doses in space will generally be protracted, and any assumption that fractionation does not alter carcinogenic response to HZE particles is subject to question. Five rats were irradiated with 10 fractions of 5cGy each, separated by approximately 20 minutes between fractions. We wish to

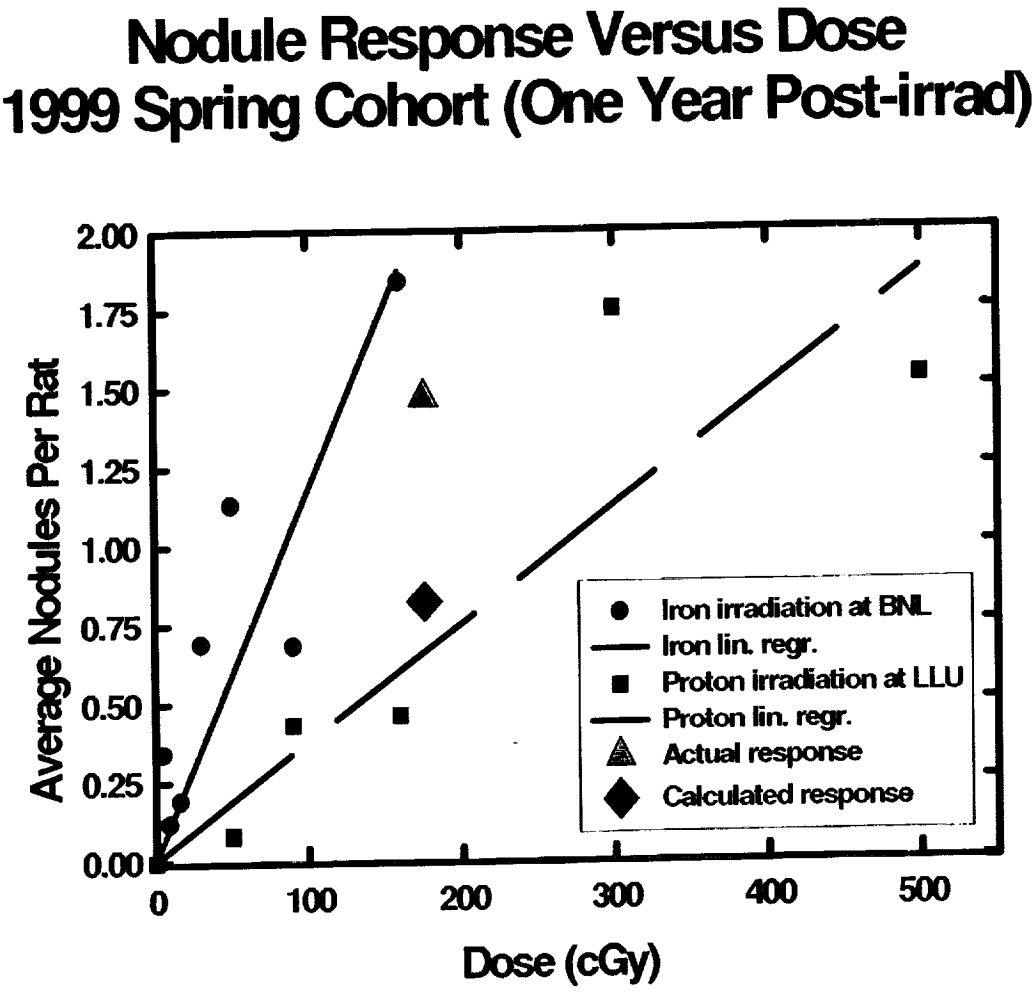


Fig. 12. A comparison of measured response for sequential irradiations with iron ions and protons with the expected additive.

compare their carcinogenic response with the acutely irradiated 50cGy animals. At the present time, too little time has passed to perform an analysis, but this group will be followed.

E. MODELING LOW DOSE-RATE RESPONSES TO THE DIFFERENT RADIATIONS

Mathematical descriptions of ionizing radiation responses provide a means for extrapolating biological models over particle type, dose or fluence-rate and accumulated dose. Successful extrapolations of existing data to risks associated with protracted HZE radiation exposures, first in rats and ultimately in humans, are necessarily going to have to be mechanistically based, at least in part, and may have to account for the genetic and molecular interactions responsible for radiation sensitivity and genomic instability, changes in cellular proliferation, and ultimately, carcinogenesis (Williams *et al.*, 1996b) In the past, mathematical models have largely been limited to hit/target models using phenomenological treatments of repair, cell turnover, and arrest. Included in these models are ones that treat the cell cycle progression and arrest using Monte-Carlo simulation (Dillehay, 1994), cell phase compartmental models using a state-vector approach (Wilson *et al.*, 1993), and maturation-age diffusion equations. The model of Wilson *et al.* (1993) uses a multi-hit kinetics formalism with the allowance of repair/misrepair and a cell phase compartmental approach for the evolution of individual cell populations into the next cell phase, including the doubling at mitosis. The model allows for both a G2 or G1 arrest by assuming damaged cells are blocked in their progression at a rate dependent on the number of hits received in the compartment. The model provides a good representation of the measurements for the evolution of cells in each phase of the cell cycle by assuming increased sensitivity of cells hit in G1 and a arrest time of about 8 hours per hit in G1. This is a straightforward approach for distinguishing between wild type or mutant p53 cell lines, which do or do not express a G1 arrest.

The construction of mathematical models that incorporate gene expression, protein interactions, and signal transduction with radiation response would provide a more mechanistic approach to model radiation damage and cell physiology. Recently, we have developed a mathematical model of G1 control that accounts for the behavior of the cyclins, cyclin dependent kinases (cdks), the pRb protein, and the transcription factor E2F/DP (Cucinotta *et al.*, 1997). Included in this model is the description of the two classes of cyclin kinase inhibitors exemplified by the p21 and p16 proteins, respectively. The new approach of Cucinotta and Dicello (2000) to consider gene expression in models of radiation sensitivity is described next. Necessarily, such an approach requires multiple parameters describing the kinetics or simplifying assumptions to reduce the number of variables. In practice, it is a combination of both of these approaches with a strong dependency upon experimental biology to continuously validate the theory to the application. In other words, such models will have the greatest accuracy when the interpolation or extrapolation is not too far from the experimental benchmark. In response to previous suggestions by reviewers, we will provide some details of this subproject.

To have the most complete set of relevant data for the Spraque-Dawley rat tumor responses, we have been closely collaborating with Williams et al. (1996) to supplement that available in the literature. We first consider experiments that demonstrate the importance of cell cycle proteins in determining radiation responses and show how these complex interactions can be incorporated into mathematical of cell cycle progression (Cucinotta *et al.*, 1997) and radiation response. We describe mathematically the molecular kinetics of the proteins that regulate radiation responses including passage through the restriction point and the G1 arrest observed in wt p53 cells following energetic photon exposures. For space radiation exposures, radiation quality is a large concern, and the models required for risk assessment must consider particle track structure (Cucinotta *et al.*, 1996) in addition to genetic

expression. Current knowledge of cell cycle progression and arrest in mammalian cells includes the description of the phosphorylation events of several protein complexes made-up of cyclins and cyclin dependent kinases (cdk) and regulation of tumor suppressor proteins. Phosphorylation delays occur during repair of damage from radiation. We have constructed our mathematical model starting with cell cycle progression in terms of the kinetics of protein complex formation and regulation.

Gene Expression and Radiation Response

Genetic expression is a major determinant of radiation sensitivity. Recently, many investigators have focused on genetic instability including the increased mutation rates, more frequent chromosome aberrations, or increased risk carcinogenesis seen in daughters of irradiated cells. Two of the most widely characterized genetic changes associated with genomic instability are p53 mutations and mismatch repair (MMR) defects. The p53 protein accumulates after DNA damage caused by many agents by the mechanism of post-transcriptional regulation. Increased levels of p53 controls the expression of other proteins, which inhibit cell cycle progression in G1 or cause apoptosis. Arrest in G1 has been postulated to provide an important protective mechanism before DNA synthesis is initiated; however many aspects of this control are understood incompletely. Repair of mismatched bases is an important mechanism for reducing replication errors. A recent characterization (Williams *et al.*, 1996b) of radiation response with several human carcinoma cell lines has characterized the response to radiation for cell lineages with three distinct genetic markers: mutant p53/MMR$^+$, wt p53/MMR$^+$, and wt p53/MMR$^-$. As shown n Figure 13, these experiments have demonstrated the increased cytotoxicity, elevated mutation frequency, and more frequent chromosome aberrations in MMR$^-$ cell lines in comparison to MMR$^+$ cells. These experiments demonstrated that MMR$^-$ (repair proficient cells) are more likely to block in G1 after protracted irradiation. Cells that possess mutated p53 do block in G2. It is observed that mutant p53 cells are more resistant to inactivation, which is attributed to a decrease in apoptosis. It is expected that genetic and molecular markers of radiation response will be continued to be studied in the future, including the role of G1 control proteins, signal transduction pathways (Mabry, 1990), and DNA repair genes.

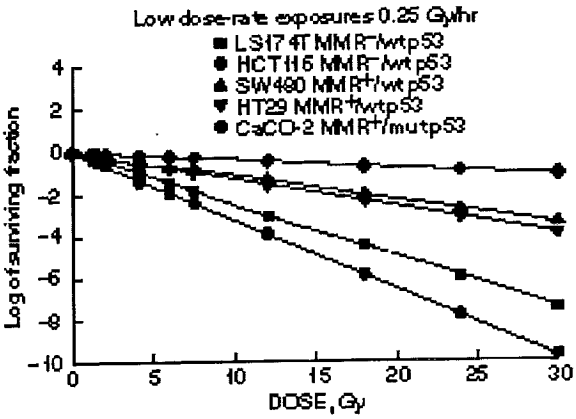


Fig. 13. Experimental survival curves from Williams et al. (1996) in several human carcinoma cell lines showing dependence of radiation sensitivity on specific genetic mutations.

Molecular Kinetics Theory of Cell-Cycle Progression

The restriction point is a protective molecular switch in the G1 phase of the cell cycle that controls whether cells divide, arrest to repair genetic damage, withdraw from the cell cycle or die through apoptosis. The proteins that control the cell cycle are of fundamental importance to understand normal growth and differentiation, carcinogenesis and the behavior of established cancers (Sherr and Roberts, 1995). Frequently, one or more of the genes that control cell cycle progression through the restriction point are mutated or epigenetically inactivated in cancers (Strauss *et al.*, 1996). These proteins form complexes that consist of threonine/tyrosine kinases known as cyclin dependent kinases (cdk's), their regulatory sub-units are the cyclins, and their inhibitors (cdki's). Progression through the cell cycle requires orderly activation and inactivation of the cyclin-cdk complexes. In Figure 14, the cell-cycle control proteins under consideration are depicted. G1 progression (Lukas *et al.*, 1994) is controlled

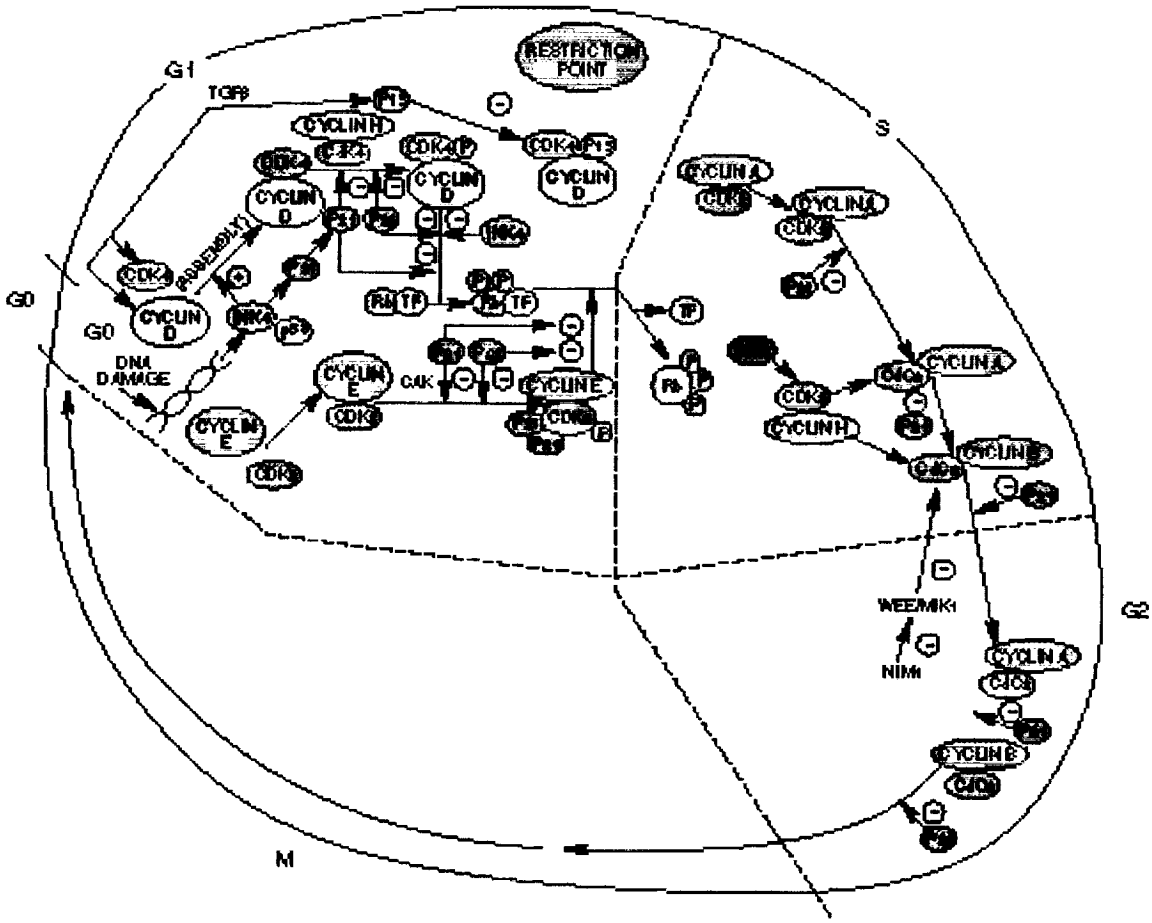


Fig. 14. Diagram illustrating functional roles of cell-cycle regulation proteins (Adapted from Hirama and Koeffler, 1995).

control proteins under consideration are depicted. G1 progression (Lukas *et al.*, 1994) is controlled by cdk4 and cdk6 and the D cyclins (D1, D2, and D3). The later G1/S checkpoint is controlled by cyclin E and cdk2 (Ohtani *et al.*, 1995, Ohtusubo *et al.*, 1995, and Koff *et al.*, 1992). S phase progression subsequently requires cyclin A binding to cdk2 and cdc2. The G2/M transition is controlled by cdc2 and members of the cyclin B family. The cyclin/cyclin-dependent kinases phosphorylate members of the Rb family (pRb, p107, and p130). After these molecules are phosphorylated, sequestered E2F-family proteins are released and initiate DNA transcription for the successful completion of the cell cycle (Welsh and Wang, 1995). Both cyclin E and cyclin A are induced strongly by E2F1, and the identity of other E2F responsive genes is currently under study (DeGregori *et al.*, 1995). The cdk inhibitors respond to external signals to control cell cycle progression by binding directly to cdks or blocking the phosphorylation of cyclin/cdk complexes (Tam *et al.*, 1994, Lukas *et al.*, 1995, and Dulic *et al.*, 1994).

The mathematical description (Cucinotta *et al.*, 1997) of G1/S control is given in Table 3. The time dependent concentrations of the cyclin, cdks, and cyclin/cdk complex's are described through their synthesis, degradation, and binding interactions.

Table 3. *Molecular Kinetics Equations for Cell Cycle Progression and Regulation. The k_{ij} are rate constants for binding of molecules i and j, v_S an $_D(v^P{}_D)$ are synthesis and degradation rates, respectively. The k^P (r^P) and k^Q (r^Q) are rates of phosphorylation or de-phosphorylation, respectively. The k_F is rate of re-formation of a pRb/E2F complex denoted [pRb-E2F]. The cyclin associated kinase complexes are labelled with superscript I = 0,1,2,P for activity of phosphorylation sites. Concentrations of molecules with P superscripts are the activated form and those with superscript 0 are the inactive forms. pRb is assumed to be de-phosphorylated by a G2/M cyclin/cdk complex.*

$$d[Cyc_i]/dt = v_{Si}[TF_i] - v_{Di}[Cyc_i] - \Sigma_j k_{ij}[Cyc_i][cdk_j]$$

$$d[Cyc^P{}_i]/dt = \Sigma_j v_{Dij}[M^P{}_{ij}] - v^P{}_{Di}[Cyc^P{}_i]$$

$$d[cdk_j]/dt = -\Sigma_i k_{ij}[Cyc_i][cdk_j] + \Sigma_i v_{Dij}[M^P{}_{ij}] - \Sigma_\alpha k_{j\alpha}[cdk_j][Ink_\alpha] + \Sigma_\alpha v_{D\alpha}[Ink_\alpha\text{-}cdk_j]$$

$$d[M^I{}_{ij}]/dt = k_{ij}[Cyc_i][cdk_j]\delta_{I0} - v_{Dij}[M^I{}_{ij}] - \Sigma_J \varepsilon_J k^{P,Q}{}_{ij}[M^J{}_{ij}] + \Sigma_J \varepsilon_J k^{Q,P}{}_{ij}[M^J{}_{ij}]$$

$$- \Sigma_\beta k_{ij\beta}[KI_\beta][M^I{}_{ij}]\delta_{I0} + \Sigma_\beta v_{D\beta}[KI_\beta\text{-}M^0{}_{ij}]\delta_{I0}$$

$$d[pRb^P]/dt = \Sigma_j r^P{}_{ij}[M^P{}_{ij}][pRb\text{-}E2F_{i+1}]/(K+[pRb\text{-}E2F_{i+1}]) - r^Q{}_{G2/M}[M^P{}_{G2/M}][pRb^P]$$

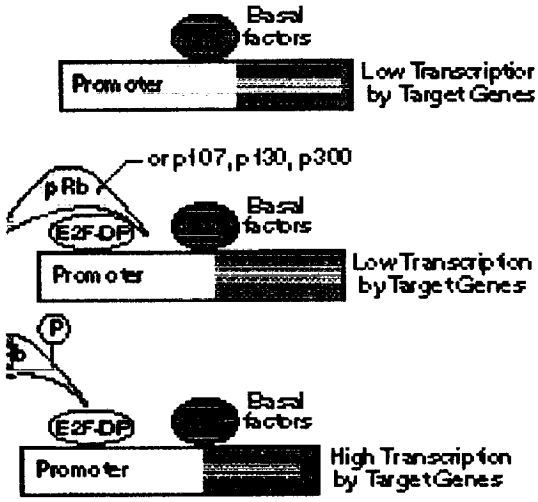
$$d[E2F_{i+1}]/dt = \Sigma_j r^P{}_{ij}[M^P{}_{ij}][pRb\text{-}E2F_{i+1}]/(K+[pRb\text{-}E2F_{i+1}]) - k_{F\,i+1}[E2F_{i+1}][pRb^0]$$

$$d[Ink_\alpha]/dt = v_{S\alpha}[TF_\alpha] - v_{D\alpha}[Ink_\alpha] - \Sigma_j k_{j\alpha}[cdk_j][Ink_\alpha]$$

$$d[Ink_\alpha\text{-}cdk_j]/dt = k_{j\alpha}[cdk_j][Ink_\alpha] - v_{D\alpha}[Ink_\alpha\text{-}cdk_j]$$

$$d[KI_\beta]/dt = v_{S\beta}[TF_\beta] - v_{D\beta}[KI_\beta] - \Sigma_{ij} k_{ij\beta}[KI_\beta][M^0{}_{ij}]$$

The bound state of the pocket protein with the E2F/DP hetero-dimers acts as a substrate of cyclin-dependent kinases. In our model, we account for the release of the E2F's from pRb by cyclin kinases leading to transcription of E2F responsive genes as depicted in Figure 15a. To model this behavior mathematically requires an undamped oscillatory solution to the non-linear rate equation's which are known as limit cycle solutions. Figure 15b depicts the regulation of the cyclin/cdk complex. The phospatase molecules of the kinases provide both negative and positive control on the kinases. We follow the G2/M control model of Novak and Tyson (1993) and use rate constants for regulation of the negative residue that have a quadratic dependence on the kinase and assume that the activity of the positive residue is in equilibrium with the de-phospatase. The degradation by ubiquitin-mediated proteolysis of the cyclins with the sequence homology known as the cyclin destruction box are assumed to have a rate-constant with a quadratic dependence on the active kinase. In Figure 16a we show the solution of the model for the time-rate of change of the D and E cyclins and the



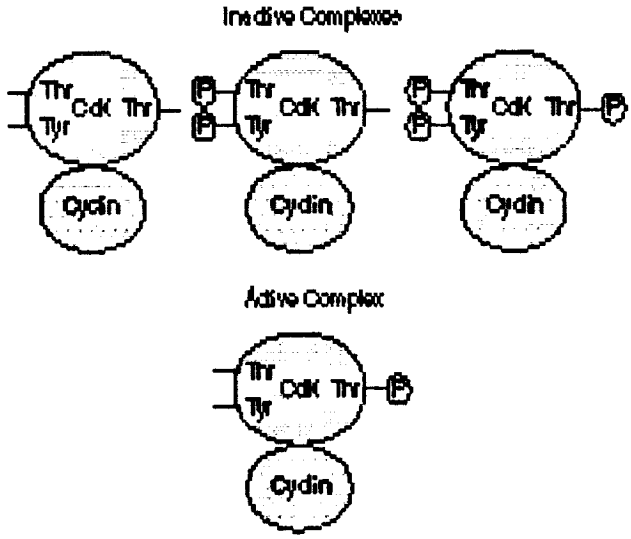
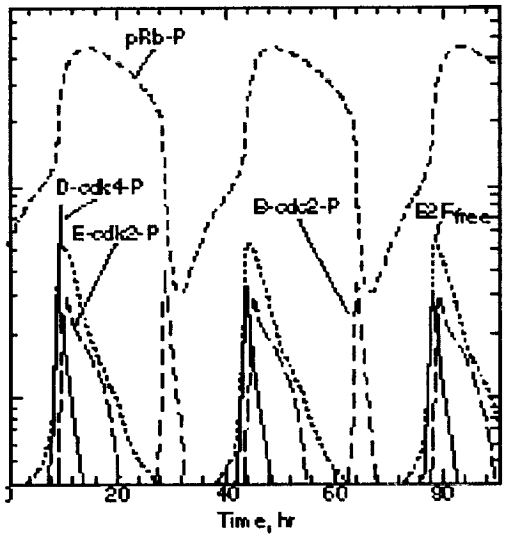


Fig. 15a. Diagram illustrating role of pRb and its phosphorylation and expression of transcription factors E2F/DP.

Fig. 15b. Diagram illustrating positive and negative controls on kinase activity of cyclin/cdk complexes.

solution of the model for the time-rate of change of the D and E cyclins and the phosphorylation of pRb. In Figure 16b we show the increase in proliferation that occurs in the model in Rb deficient cells because of a decrease in G1 duration when E2F's are not regulated by pRb.

P53 Regulation After DNA Damage



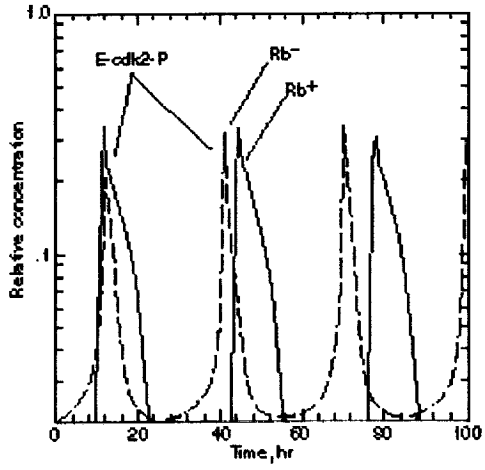


Fig. 16a. Calculations of cyclin E kinase in normal and Rb- cell lines showing proliferation (shortened G1 times).

Fig.16b. Calculated percent expression of the cyclins D, E, and B as a function of time over several cell cycles showing limit cycle behavior found in the model.

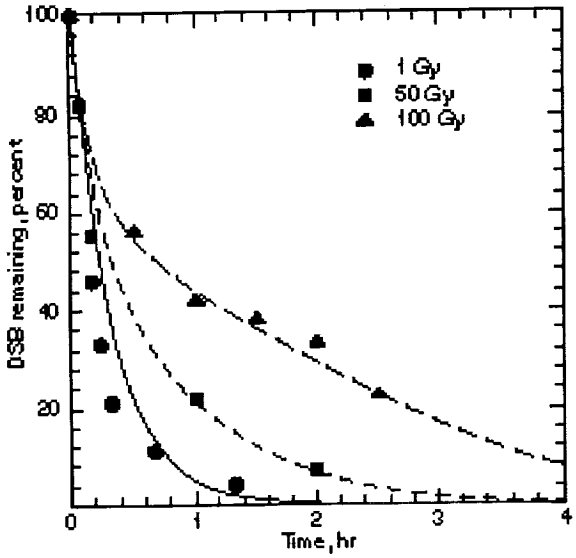


Fig. 17. Calculations of DSB repair versus time after exposure. Also shown are DSB repair data of McWilliams et al., 1983, and G. P. Van Der Schans et al., 1983.

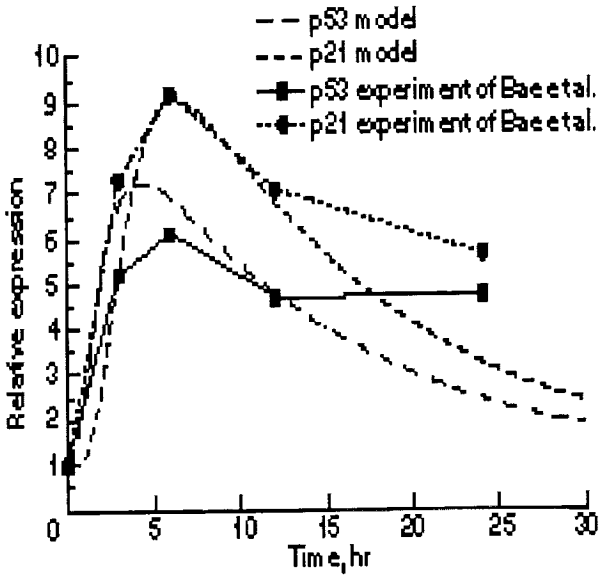


Fig. 18. Model calculations of p53 and p21 expression versus time after 6.3 Gy acute exposure as fit to data of Bae et al., 1995.

which also produces strand break formation by a post-transcriptional mechanisms. (Nelson and Kastan, 1994). To consider p53 regulation in the model we consider the coupling of p53 directly to an enzyme complex for DSB repair. Single strand breaks (SSB) will also regulate p53; however, since many SSB's are rejoined directly by ligases or repaired rapidly, we will model the longer lived DSB repair complex in our calculations. The half-life of p53 is 25 minutes. Coupling of the molecule to DNA damage will increase this signal by several hours depending on the level of damage and other modifying events. These include phosphorylation of p53 by molecules that regulate its DNA binding activity and proteolysis. Molecules responsible for phosphorylation of p53 include raf, DNA-PK, PKC, and cdk-cyclin complex's (Steegenga *et al.*, 1996). The latter may be involved in a feedback control mechanism, since the inhibition in activation of several cyclin associated cdk complexes is through transcription of p21 by p53.

Following irradiation, the number of DSB's for energetic photon exposure occurs at the rate of 20–50 DSB/Gy. A heuristic model for the post-transcription modification of p53 is made by coupling p53 to the DNA repair complex. The repair complex is formed by radiation induced DSB's as

$$\text{radiation} + [\text{DNA}] \rightarrow [\text{DSB}] \tag{1}$$

$$[\text{DSB}] + [\text{E}_{\text{rep}}] \rightarrow [\text{C}_{\text{rep}}] \rightarrow [\text{DNA}] + [\text{E}_{\text{rep}}] \tag{2}$$

The regulation of p53 through the DSB repair complex, $[\text{C}_{\text{rep}}]$, is described as

$$d\,[\text{p53}]/dt = v_S - v_D\,[\text{p53}] - k_{\text{p53}}\,[\text{p53}][\text{C}_{\text{rep}}] - \{\text{other interactions}\} \tag{3}$$

An effective half-life of p53 can then be defined as

$$1/\tau_{\text{p53}} = v_D + k_{\text{p53}}\,[\text{C}_{\text{rep}}] \tag{4}$$

which leads to a dose dependent increase of the half-life over the base line value. We are ignoring the kinetic step of tetramerization of p53 since it occurs rapidly in several minutes or less. The repair of DSBs proceeds with first-order kinetics at low doses and a mixture of first and zeroth-order kinetics at higher doses as the number of repair enzymes becomes comparable to the number of break's (Lett 1994). In Figure 17 we show the comparison of the model to DSB repair data (McWilliams *et al.*, 1983 and G. P. Van Der Schans *et al.*, 1983). The enzyme repair model gives a good representation of the data for the doses shown. Several future considerations of the model include the possibility that the expression of the repair enzymes is regulated by the exposure such that exposures above about 0.5 Gy are not indicative of the repair system at lower doses or dose-rates studied here. Although p53 regulation has been shown to involve damage recognition of strand breaks (Lu and Lane, 1993, Nelson and Kastan, 1994, and Reed *et al.*, 1995), its regulation needs to studied as a function of cell cycle position and radiation type.

G1 Arrest Through the Kinase Inhibitor p21

The kinase inhibitor p21 is normally found in quaternary complexes with the cyclins, cdk's, and proliferating cell nuclear antigen (PCNA) (Namba *et al.*, 1995 and Li *et al.*, 1994). Increases in p21 concentration cause G1 arrest (Dulic *et al.*, 1994) through inhibiting G1 cyclin associated kinase activity. In our model we examine p53 dependent p21 upregulation after DNA damage as described by

$$d\,[mRNA]/dt = r_T + r_{p53}\,[p53] - r_D\,[mRNA] \qquad (5)$$

where r_T and r_D are the basal rates of transcription and degradation, respectively, and r_{p53} is the rate constant for coupling of the transcription factor p53 to the p21 promoter. The time rate of change of p21 is given by

$$d\,[p21]/dt = v_S\,[mRNA] - v_D\,[p21] - k_I\,[p21]\,[M^0_D] - k_I\,[p21]\,[M^0_E] \qquad (6)$$

where v_S and v_D are the synthesis and degradation rates of p21, respectively. The last two terms in Eq. (6) are the coupling of p21 to the G1 cyclin-cdk complexes.

For the background levels of p21 we use its known half-life of 30 minutes and assume that there is normally about one molecule of p21 in each of the G1 cyclin complexes. Fits of the model to the data of Bae *et al.* (1995) for the time course of p21 and p53 expression after the exposure for normal lymphoblast cells are shown in Figure 18. Modifications of the present model will be required to describe the modulation of these proteins for time courses greater than 10 hrs after DNA damage, including the role of phosphorylation or other conformation changes of p53 throughout the cell cycle that are known to affect its DNA binding and transcription activity. Also, genomic instability after radiation exposure would likely modify p53 levels because of the observed high sensitivity of

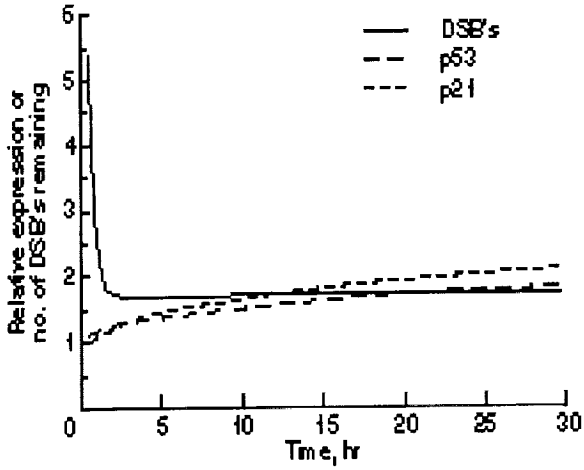


Fig. 19. Predictions of model for number of DSBs remaining and relative expression of p53 andp21 for 0.1 Gy/hr gamma irradiation.

p53 to DNA damage (Nelson and Kastan, 1994). In Figure 19 we show the number of DSB remaining and the relative expression of p53 and p21 versus time for low dose-rate exposures. These low dose-rate results predict that there is no threshold for p53 induction in agreement with the review of Lane (1996) for low dose-rate acute exposures.

In Figure 20 we show comparisons of the model for acute exposures of 6.3 Gy in early G1 and at G1 + 6hrs. The results in Figure 8a display a large G1 arrest in agreement with experiment (Dulic *et al.*, 1994). The results of the model shown in Figure 20a are in general agreement with the Western blot analysis of Dulic *et al.* (1994). The results of Figure 20b show only minimal G1 arrest for the same exposure level. Here, the non-linear expression of kinase activity prevents the inhibitor in causing arrest. These results suggest that for asynchronous populations, G1 arrest is dependent on timing of signaling pathways and non-linear accumulation of cyclin associated kinases.

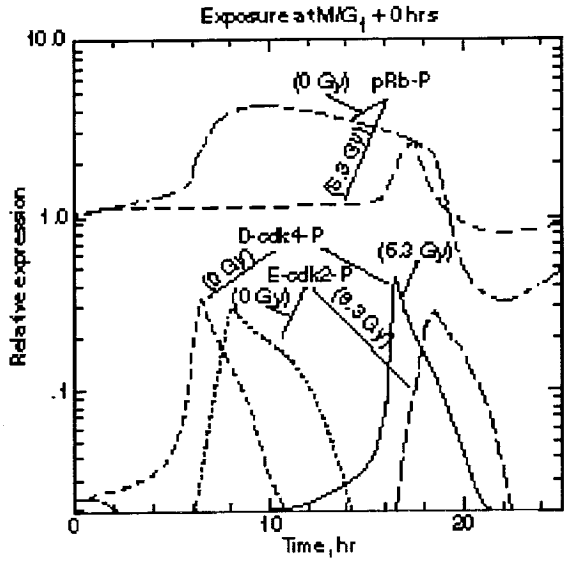


Fig. 20a. Calculations of relative expression of several proteins after acute exposure of 6.3 Gy at M/G1 border.

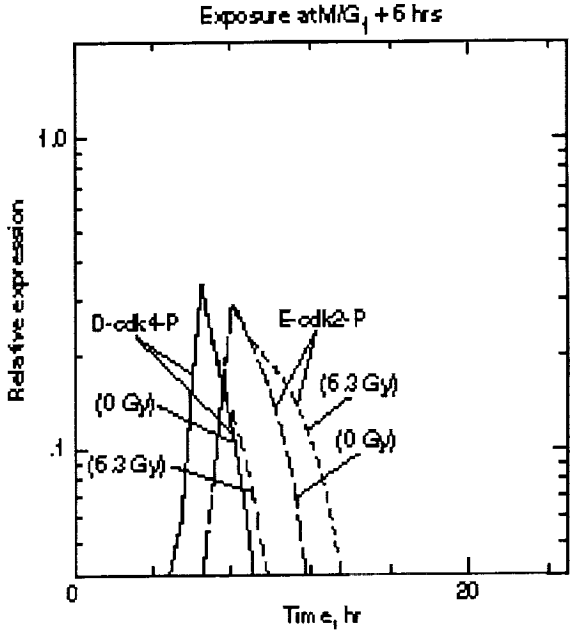


Fig. 20b. Same as Fig. 8a except it is exposed at M/G1 border plus 6 hrs.

Outlook

Modification of intracellular signaling and its effect on cell cycle control is a potential determinant in radiation response and must be adequately understood for extrapolating risk models to low dose-rates and complicated radiation fields. The low dose-rate experiments of Williams *et al.* (1996b) highlight the importance of genetic expression in several human carcinoma cell lines. We have described a mathematical model of cell cycle control through regulation of the related proteins and the coupling of this model to DNA damage through the p53 signal transduction pathway. In future work the model discussed here will be extended to describe exposures to asynchronous cell populations. The role of p53 regulation by DNA damage and many hours after exposure, and the description of the G2 arrest will be determinants in this description. Other aspects of p53 regulation than described here will be

considered. A similar mathematical description can be applied to study the early events in the apoptosis pathway (Zhan *et al.*, 1994), including p53 induction of BAX and BAX formation of hetero-dimers with members of the Bcl family.

In-Vivo Modeling of Tumorigenesis

We have devised a mathematical model of the interactions of cell cycle control proteins including cyclins, cdk's, pRb, E2F's, cdk inhibitors, and growth factors. The model has the potential for describing complex multstage processes such as cancer pathways. We have already applied it to calculate mechanistic-based cancer-risks for different tumors for the different radiation types and exposures of interest. As outlined in Fig. 21, Russo and Russo (1987, 1996) and Russo et al. (1983) characterized differentiation in the rat mammary gland and its response to a chemical carcinogen, DMBA. We use our mathematical model of molecular events in cell cycle progression to develop a quantitative model that characterizes differentiated structures of the mammary gland and initiation in control populations. We apply this model to describe the growth kinetics of undifferentiated structures in the rat mammary gland. The progression stage is believed to result from a differentiation of terminal end buds (TEBs) into alveolar buds (ABs) and lobules. Alternatively, the terminal end buds can regress to terminal ducts (TDs) as seen with aging. The pathways leading to both benign and malignant tumors are illustrated schematically in Figure 21. Young virgin rats are known to have the highest cancer risk. This increased sensitivity is attributed to their larger number of TEB's which possess a characteristicall y short G1 phase, and the

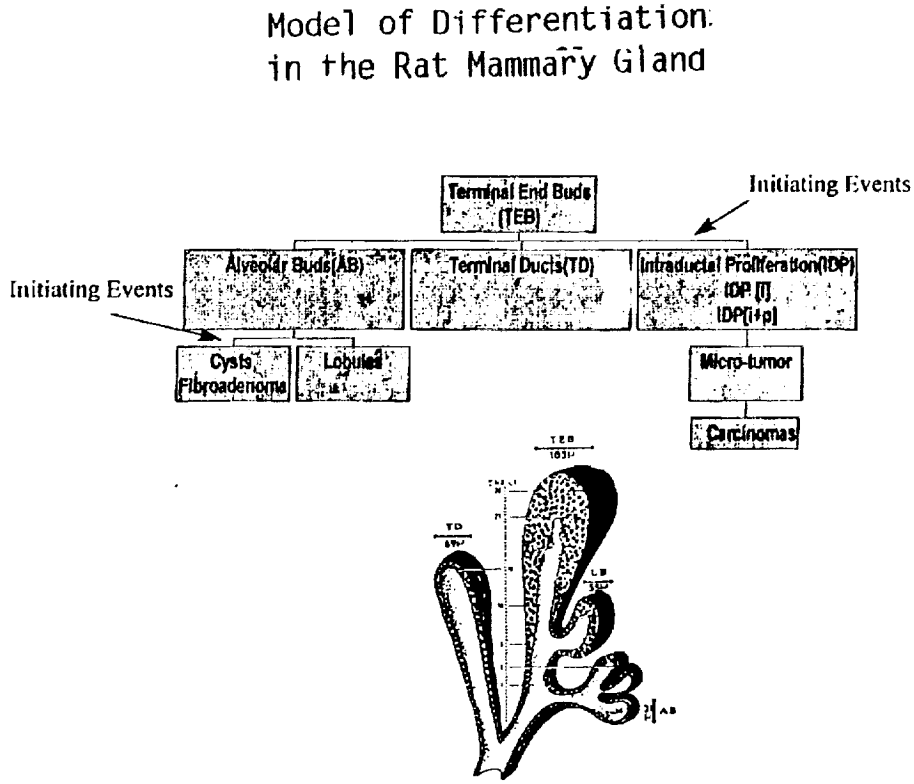


Fig. 21: A schematic of the pathways underlying the mathematical model used to simulate mammary-tumor induction in the Spraque-Dawley rat, according to the paradigm of Russo et al., (1983).

higher sensitivity of S phase cells. The model is based on rate limiting transitions manifested in the activation of cyclin-cdk complexes through covalent modification and controls on cyclin transcription. We simulate the uncontrolled expansion of undifferentiated structures in the mammary gland using known mutational events that alter cell cycle controls, including pRb, p16 mutation and cyclin D over-expression.

The model assumes that entry into the cell cycle is stimulated by mitogen activation of cyclin D, and down-stream activation of cyclins E, A, and B with transcription control by E2F's sequestered by pRb. Application of this model to observations of different cell lineages in the rat mammary gland was achieved by modifying control of cyclin D expression in order to modify the length of G1 phase.

The same non-linear differential equations describing this model are shown in Table 3 and are applied to the mammary gland using the rate constants and the cell-cycle parameters, presented in Tables 4 and 5, which were determined from data in the literature.

Table 4. *Model Rate Parameters and Experimental Values. Model rate parameters are given for synthesis and degradation of cyclins and p16 and exp. degr. constants based on reported ½ lives. Degradation rates for cyclins are for activated cyclin/cdk complex.* $r^P = 5\ hr^{-1}$ *and* $r^Q = 0.1\ hr^{-1}$ *for cyclin/cdk phosphorylation of [pRb-E2F]. Other rate constants;* $k^P_2 = 180\ hr^{-1}$ *and* $k_F = 0.6\ hr^{-1}$ *for each i,* $k_{p16,cdk4} = 12\ hr^{-1}$.

Protein	[TFi]	$_{vs,}$ model	$_{vd,}$ model	$_{vd,}$ expt.	k^p_o
[Cyclin D]					
-TEB	mitogens	0.5,hr^{-1}	½, hr^{-1}	½, hr^{-1}	0.03, hr^{-1}
-TD	mitogens	0.15	½	½	0.03
-AB	mitogens	0.1	½	½	0.03
[Cyclin E]	[E2Fe]	1.08	1/3	-	0.015
[Cyclin A}	[E2Fa]	0.38	1/4	1/4	0.015
[Cyclin B]	[E2Fb]	0.53	0.075	0.067	0.09
[p16]	[TF_{16}]	1.1	0.125	<1/3	-

Table 5. *Cell-Cycle Parameters from Russo and Russo ('1987)*

Group	Structure	Gl, hr.	S, hr.	G2, hr	M, hr.	T_{cycle},hr	Growth Fraction
Young Virgin	TEB	.72+/.42	7.2+/.80	1.08+/.11	1.2+/.2	9.93+/.31	0.55
	TD	7.5+/.12	7.5+/.83	1.00+/.01	1.4+/.4	17.33+/.42	0.39
	AB	17.3+/1.1	8.7+/.61	1.00+/-.01	1.2+/.2	28.2+/1.98	0.13
Old Virgin	TEB	5.2+/.99	7.6+/.40	4.40+/-.40	1.6+/.2	18.8+/0.99	0.077
	TD	7.1+/.08	7.6+/.40	4.40+/-.40	1.4+/.2	20.57+/1.8	0.020
	AB	17.3+/1.4	8.2+/.72	3.60+/-.40	1.7+/.1	30.75+/-2.7	0.008

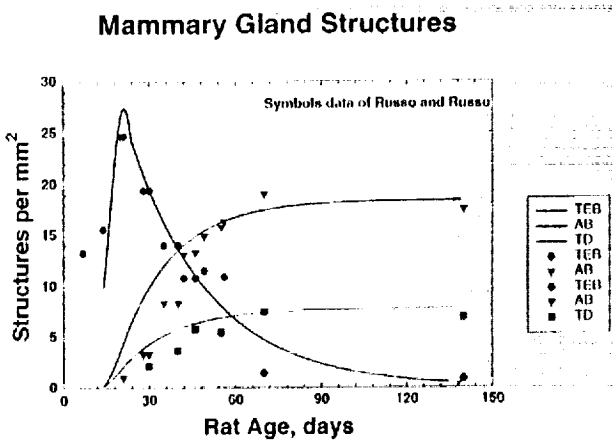
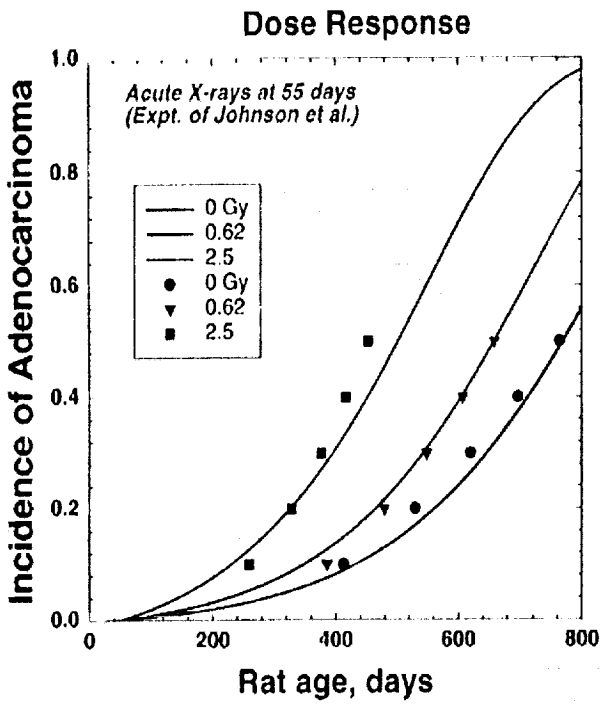


Fig. 22: A comparison of the theoretical model (solid lines) with the experimental results (symbols) for the progression and regression of differentiation of the rat mammary gland for the terminal end buds (TEBs), the alveolar buds (ABs), and the terminal ducts (TDs).

We have applied the model to a situation of importance in designing our experiments, but for which there are insufficient data available. Because of the logistics of moving the animals and personnel to and from the various facilities used for the irradiations and the impossibility of doing all of the irradiations simultaneously, it is essential to understand how significant are the variations in response with the age of the animals at the time of irradiations. Figure 22 illustrates that the model is capable of adequately describing the observed variations in the number per unit volume of the critical structures with time as measured by Russo and Russo (1987, 1996). Then the results of the model for the variation in the development of mammary carcinomas as a function of time and dose for photon irradations are compared with the data of Johnson et al., (1989) in Figure 23. The incidence rate of the Sprague-Dawleys as a function of age was calculated and is presented in Figure 24. There are no data in the literature, so these were truly predictive results, results which we needed to determine the number of animals per

dose point, when to irradiate, and how much time variation we could tolerate, knowing the large accelerator facilities are unreliable for meeting schedules set months in advance so we could order animals.



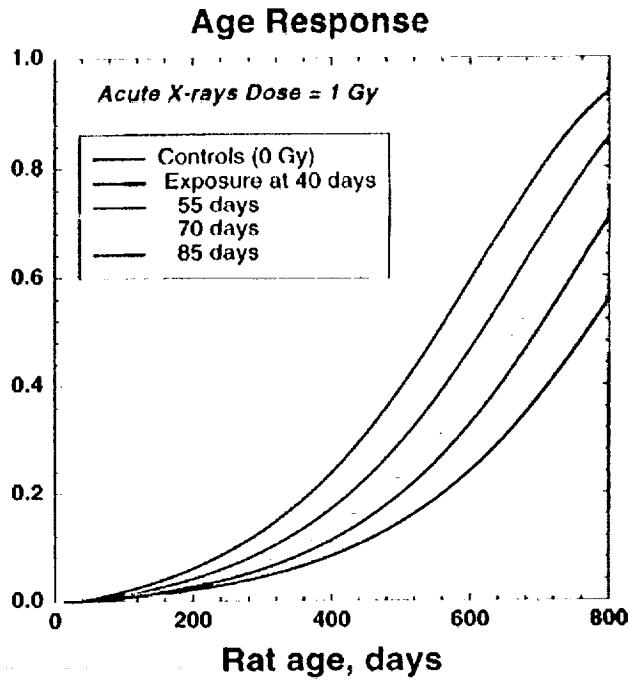


Fig. 23: A comparison of the incidence of adenocarcinomas in the rat mammary gland for the theoretical model (solid line) and the experimental results (symbols) of Johnson et al. 1989).

Fig. 24: An estimation of the change in the incidence rate as a function of age of the rats at the time of exposure of 1 Gy of photons.

Finally, preliminary results of a maximum likelihood analysis which we (Lief Peterson, Baylor, Cucinotta, and Dicello) are in the process of developing are presented in Figures 25a and 25b. This analysis is based upon a proportional-hazards model for relative risks of mammary tumors based and accounts for all radiation types and dose levels. Survival experience among cesium dose groups form the baseline for the survival experience.

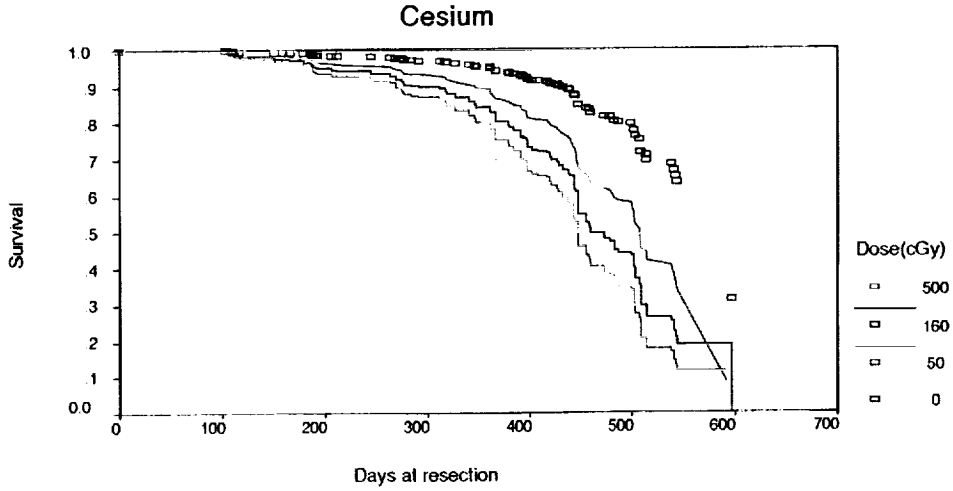


Fig. 25a. Preliminary results of a maximum likelihood analysis for relative risk for tumor-free survival as a function of time after irradiations with cesium-137 gamma rays. The cesium groups formed the baseline.

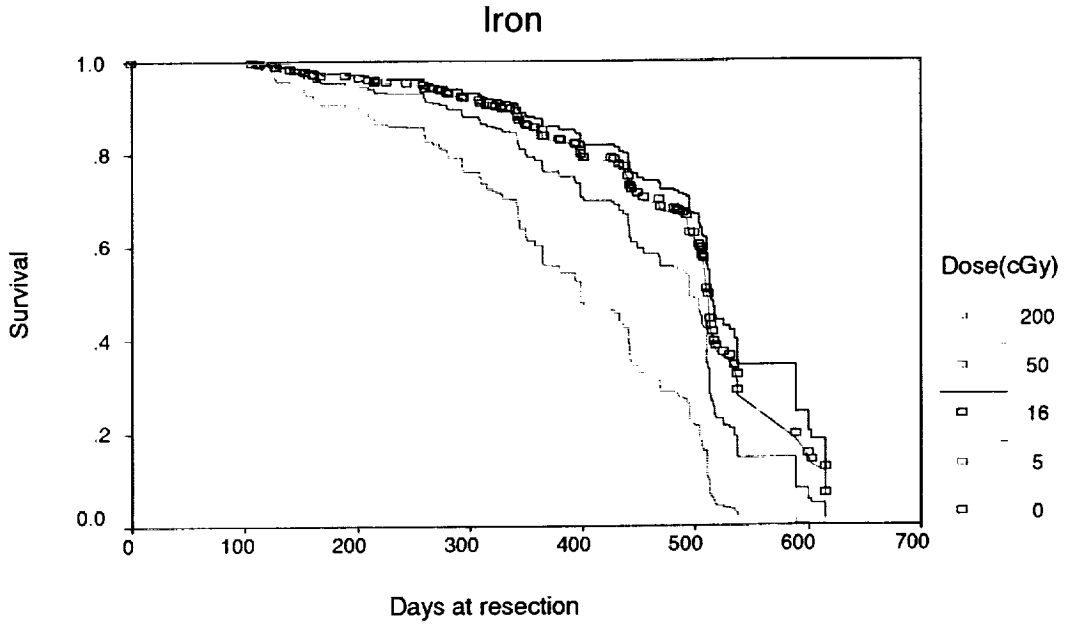


Fig. 25b. Preliminary results of a maximum likelihood analysis for relative risk for tumor-free survival as a function of time after irradiations with iron ions.

II. IMPLICATION OF PROJECT FINDINGS FOR FUTURE RESEARCH

The goal of our research at the end of the next three-year period is to be able to extrapolate carcinogenic risks in these animals to human risk in space. As noted in our original proposal, the animal studies will take about five years to complete. A fraction of the animals from the first cohort, irradiated during the second month of this project three years ago, are still alive. A significant number of animals from the most recent irradiations during this past year are expected to live for another three years. The animals receiving the lowest doses, the most interesting doses in terms of space applications, are expected to show differences in comparison with the sham-irradiated rats only in the latter years. One of our main goals, then, must be to follow the previous experiments to completion.

Despite the fact that the carcinogenesis studies are incomplete:

1. We have obtained absolute risks for 1-GeV iron ions, protons, and photons for mammary carcinomas and pituitary cancers resulting from whole-body exposures. As a result of a judicious choice of doses, we also have determined relative doses that produce equivalent responses (RBEs).
2. We have obtained the first such data for tissues that exist in humans , primarily breast and pituitary.
3. The data obtained are for an out-bred strain and a tissue site that has been successfully extrapolated to human applications.
4. In collaboration with the Chemoprevention Project, we have shown that reasonable doses of drugs can reduce the incidence of cancers in the case of heavy-ion irradiations even at relatively low doses.
5. The data suggest that differences in response to the drugs in comparison with the photon control may exist even in the case of protons-irradiated animals. That is, the response to photons to a specific drug may not characterize the proton response (Likewise, the number and types of chromosome aberration were not equivalent as well).

Having established the credibility of our initial hypotheses, the next step must be to find the most useful drugs or dietary supplements. The results show that Tamoxifen may be an effective chemopreventor for breast cancers. Although estrogen receptor modulators (SERMs) may be effective not only for breast cancers but possibly for other sites as well. However, it was not our intention that SERMs be the ultimate or universal pharmaceutical.

The next round of experiments should be directed toward determining drugs or dietary supplements that are more generic and less hormone dependent for their path of action.

We propose as the next course of action:

1) Complete two remaining major experiments with the Sprague-Dawleys because of the good data base, namely a) a fractionation study with protracted doses of protons and iron, and b) sequential irradiations with both protons and iron.
2) Switch to a relevant mouse model that can relate to the earlier rat results. We propose the Min-mouse model, because it is a natural mutation that has been used extensively for colorectal studies, one of the most relevant tissues for radiation exposure. Because it is a nautural mutation, this model avoids the problems associated with extrapolating results with trangenic strains to humans.
3) Examine a limited number of dietary supplements such as Bowman-Birk inhibitor (BBI), for their ability to reduce the incidence of radiation-induced cancers.

4) Do a similar study with Sulindac, the most studied therapeutic drug under investigation for colorectal cancers.
5) Examine CNS damage in the same animal model, because CNS damage remains the largest unresolved issue beyond cancer.
6) We believe that the data for the Japanese atomic-bomb survivors may be available for reanalysis on an individual basis, which has never been done before. This would allow an analysis of correlative diseases and a more detailed epidemiological review. Because these data remain the necessary link to human responses, such an analysis has major potential in reducing the uncertainties of risk calculations in space.
7) Finally, we have sufficient data to begin a comprehensive risk analysis first for the animal experiments and then extrapolating to humans.
8) Because any extrapolation to humans will likely be mechanistically based, we must continue to obtain cellular, cytogenetic, and genetic information directly related to the animal studies and their human counterparts.

We have proposed a comprehensive study to NSBRI that addresses these issues over a three-year period, and it is available for further information.

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APPENDIX B: List of Publications Supported Through NSBRI Funding

1. F. A. Cucinotta and J. F. Dicello, "On the Development of Biophysical Models for Space Radiation Risk Assessment." Adv. Space Res. 25, 2131-2140 (2000)
2. J. R. Williams, Y. Zhang, H. Zhou, M. Osman, D. Cha, R. Kavet, F. Cucinotta, J. F. Dicello, L. E. Dillehay, "Predicting Cancer Risks in Astronauts from Animal Carcinogenesis Studies and Cellular Markers." Mutation Res. 430, 255-269 (1999).
3. M. Zaider, J. F. Dicello, and Y. Horowitz, "Where Do We Stand on Solid State Microdosimetry?" Radiat. Prot. Dos. 82, 163-166 (1999).
4. Task Group for "A Strategy for Research in Space Biology and Medicine in the New Century." National Research Council/National Academy of Sciences, National Academy Press, 1998.
5. R. Setlow, Dicello, J.F., Fry, R.J.M., Little, J.B., Preston, R. J., Smathers, J.B., Ullrich, R.L. Task Group on the Biological Effects of Space Radiation. Radiation Hazards to Crews of Interplanetary Missions: Biological Issues and Research Strategies. National Research Council/National Academy of Sciences, National Academy Press, 1996..
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7. J.F. Dicello, M. Zaider, and M. N. Varma, "The Correlation of Microdosimetry with Radiation Effects: An Inductive Approach." Adv. Space Res. (10)899-(10)910 (1994).
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12. F. Dicello, J. T. Lyman, J. C. McDonald, and L. J. Verhey, "A Portable System for Microdosimetric Intercomparisons by Task Group #20 of the American Association of Physicists in Medicine (AAPM). Nucl. Inst. Meth. B45, 724-729 (1990).
13. M. Zaider, J. F. Dicello, and J. J. Coyne, "The Effects of Geometrical Factors on Microdosimetric Probability Distributions of Energy Deposition." Nucl. Instr. Meth. 40/41, 1261-1265 (1989).
14. L. J. Peters, H. R. Withers, K. A. Mason, and J. F. Dicello, "Effects of Fractionated Doses of Pions on Normal Tissues: Part I3. Mouse Jejunum," Int. J. Radiat. Oncology, Biol. Phys. 6, 1667-1669 (1981).

APPENDIX C: List of Grant Submissions

1) Principal Investigator $500K 1/1/00-12/31/01
with J. Lombardo ($215K to SOM, Dicello, PI; $285K to APL, Lombardo, PI)
5% effort Rad. Onc. salaries, fringes, & indirect: $209K
Research Grant: "Pilot Project Proposal: Remote Treatment Planning for Oncology Patients or Remote Intensive Care Support"
Funding Agency: State of Maryland
Comments: *A competitive award to develop a network system for planning treatments at Johns Hopkins for cancer patients being treated in rural Maryland and interactively review those treatments with the physician and personnel at the rural care center.*

2) Co-Principal Investigator $1.4M/3years 12/1/99-11/30/01
(with subcontract to JHMI SOM for $353,960, Dicello, PI)
10% effort Rad. Onc. salaries & fringe: $353K
Phase II Research Grant: "Biomedical Applications for the Next Generation Internet (NGI): Radiation Oncology Treatment/Care Delivery Application"
J. Lombardo, P.I.)
Funding Agency: NIH/NLM
Comments: *A competitive award for a Phase-II study of the previous pilot study.*

3) Co-Investigator $5.5M/4years 9/1/78-7/31/2002
0% effort
"Research Training in Use of Tracer Principles in Oncology".
NCI training grant.
(H.Wagner, P.I.: 5T32CA09 199-17)
Funding Agency: National Institutes of Health/National Cancer Institute
Comments: *Training grant in School of Hygiene and Public Health under which our Ph.D. degrees in Medical Physics and M.S. degrees in Medical and Health Physics are granted. One graduate student working on NSBRI research is partially funded by this grant.*

4) Program Team Leader $51K/year [Team Grant: $ 1M] 10/1/97-9/30/00
10% Hopkins salaries & fringe: $48K
Management Award: NSBRI Radiation Effects Team
Funding Agency: NSBRI
Comments: *An administrative award for the Program Lead for a study of cancer prevention for environmental exposures to radiations for personnel in space.*

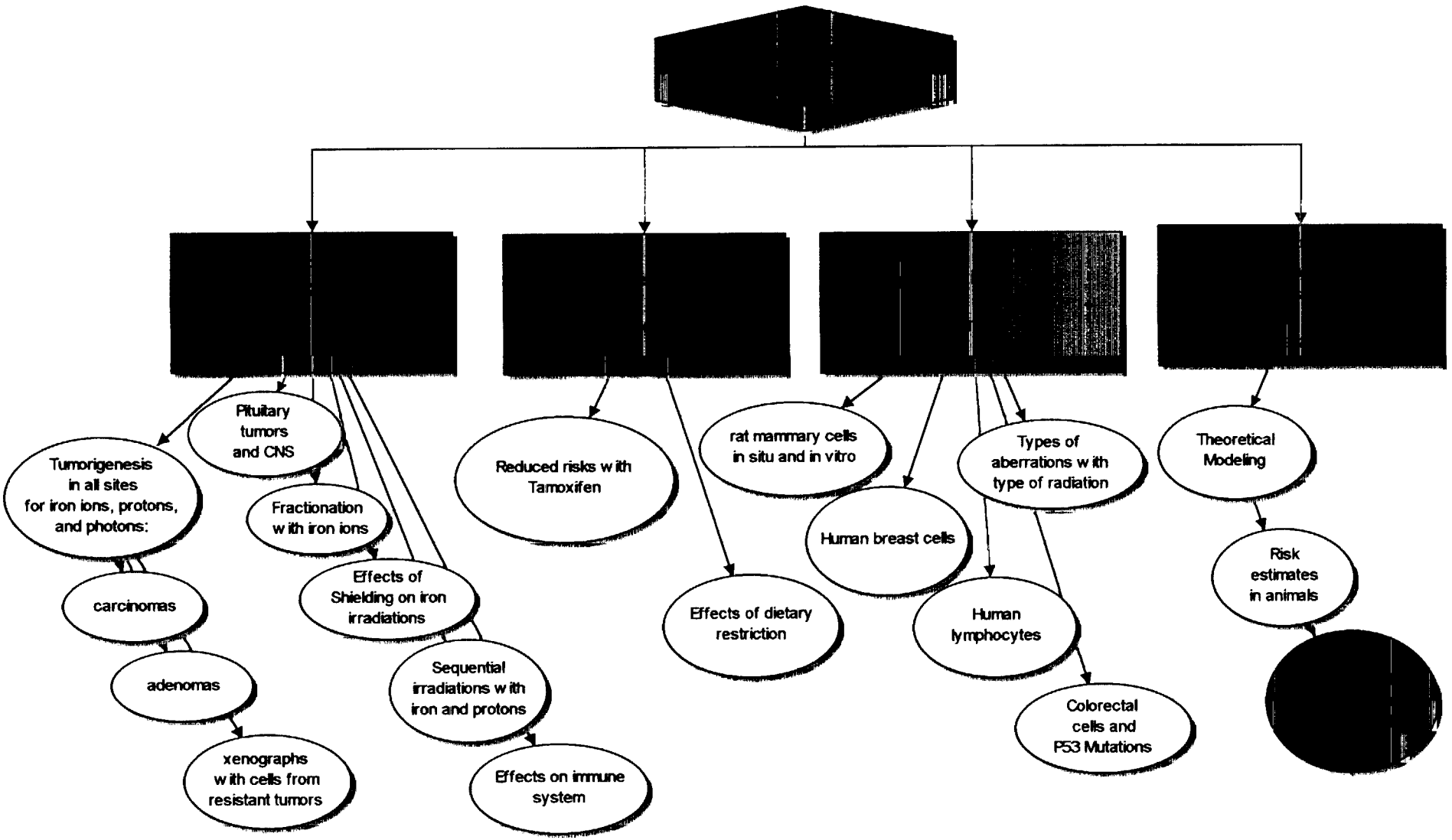
5) Principal Investigator $555K/yr not including subcontracts 10/1/97-9/30/00
40% effort Hopkins salaries & fringe: $153K
Does not include an approved rollover of $200K and an requested supplement of $100K.
Research Grant: "Radiation Effects: Core Project"
Funding Agency: NSBRI

Comments: *A competitive award for a rat study to evaluate cancer prevention from environmental exposures to photons, protons, and heavy ions through pharmaceutical intervention.*

6) Co-Investigator $280K/year 10/1/97-9/30/00
5% effort
Research Grant: "In-Situ Spectrometry of Neutrons" (R. Maurer, P.I.)
Funding Agency: NSBRI
Comments: *Secondary neutrons from high-energy therapy beams and from environmental sources represent a significant risk of cancer.*

7) Co-Investigator $5.5M/5.5years (final budget in negotiation) 11/1/99-4/30/05
10% effort
Research Grant "Martian Neutron Energy Spectrometer (MANES) for the Mars 2003 Lander Mission" (R. H. Maurer, PI)
Funding Agency: NASA
Comments: *Because of our ground-breaking research with APL in measuring neutron spectra, we were in a strong position to propose a study of the neutron background on a Mars mission and to measure that background to establish the cancer risk for such a trip.*

Original Research Structure
for
the Radiation Effects Core Project



Irradiated Female Sprague-Dawley Rats in Study

	May-98	May-99	Nov-99	Apr-00	TOTAL
Cesium (sham) @ BNL	36	20	40	0	96
Cesium (50 rad) @ BNL	36	20	40	0	96
Cesium (90 rad) @ BNL	0	20	0	0	20
Cesium (160 rad) @ BNL	35	20	40	0	95
Cesium (300 rad) @ BNL	0	20	20	0	40
Cesium (500 rad) @ BNL	36	20	20	0	76
Cesium (900 rad) @ BNL	0	20	0	0	20
Iron (sham) @ BNL	70	40	60	0	170
Iron (5 rad) @ BNL	70	40	60	0	170
Iron (9 rad) @ BNL	0	40	0	0	40
Iron (16 rad) @ BNL	71	40	60	0	171
Iron (30 rad) @ BNL	0	40	40	0	80
Iron (50 rad) @ BNL	69	40	25	0	134
Iron (90 rad) @ BNL	0	40	0	0	40
Iron (160 rad) @ BNL	0	40	0	0	40
Iron (200 rad) @ BNL	18	40	0	0	58
Iron 16 + Proton 160	0	11	0	0	11
Proton (sham) @ LLU	36	48	0	20	104
Proton (50 rad) @ LLU	36	56	0	50	142
Proton (90 rad) @ LLU	0	56	0	60	116
Proton (160 rad) @ LLU	36	48	0	30	114
Proton (300 rad) @ LLU	0	48	0	0	48
Proton (500 rad) @ LLU	36	48	0	0	84
Proton (900 rad) @ LLU	0	48	0	0	48
Cobalt (sham) @ LLU	18	20	0	20	58
Cobalt (50 rad) @ LLU	18	20	0	58	96
Cobalt (90 rad) @ LLU	0	20	0	60	80
Cobalt (160 rad) @ LLU	18	20	0	32	70
Cobalt (300 rad) @ LLU	0	20	0	0	20
Cobalt (500 rad) @ LLU	18	20	0	0	38
Cobalt (900 rad) @ LLU	0	20	0	0	20
Cesium (sham) @ JHU	18	20	0	0	38
Cesium (50 rad) @ JHU	18	20	0	0	38
Cesium (90 rad) @ JHU	0	20	0	0	20
Cesium (160 rad) @ JHU	18	20	0	0	38
Cesium (300 rad) @ JHU	0	20	0	0	20
Cesium (500 rad) @ JHU	30	20	0	0	50
Cesium (900 rad) @ JHU	0	18	0	0	18
TOTAL	741	1141	405	330	2617

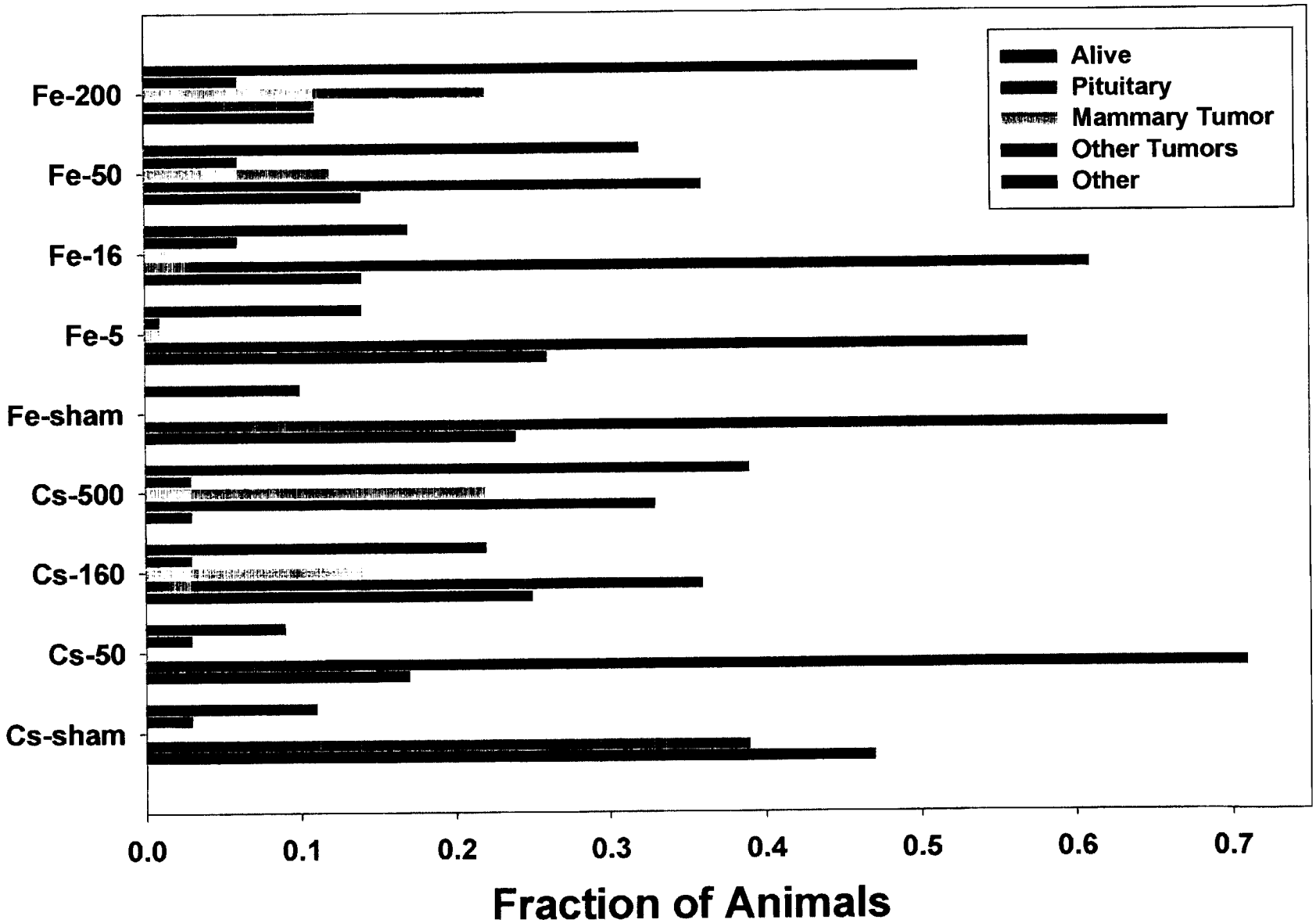
* For chemoprevention studies, approximately one-half of the May-99, Nov-99 and Apr-00 rats were administered Tamoxifen.

Number of Rats Still Alive in each Category as of 9/13/00

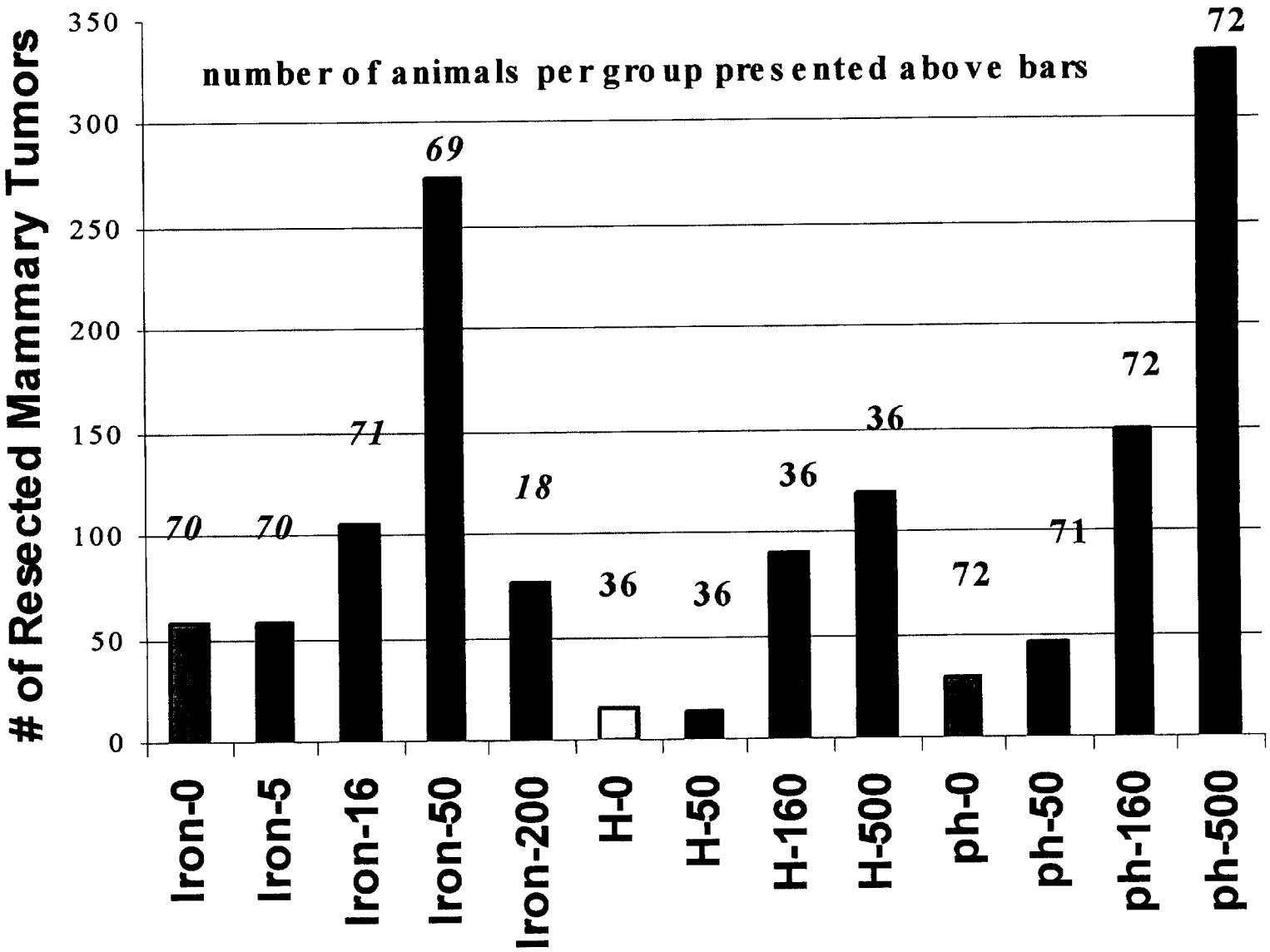
	May-98	May-99	Nov-99	Apr-00	TOTAL
Cesium (sham) @ BNL	9	18	40	0	67
Cesium (50 rad) @ BNL	0	20	40	0	60
Cesium (90 rad) @ BNL	0	18	0	0	18
Cesium (160 rad) @ BNL	2	18	39	0	59
Cesium (300 rad) @ BNL	0	17	20	0	37
Cesium (500 rad) @ BNL	1	18	19	0	38
Cesium (900 rad) @ BNL	0	15	0	0	15
Iron (sham) @ BNL	7	36	59	0	102
Iron (5 rad) @ BNL	4	35	60	0	99
Iron (9 rad) @ BNL	0	34	0	0	34
Iron (16 rad) @ BNL	1	33	59	0	93
Iron (30 rad) @ BNL	0	37	40	0	77
Iron (50 rad) @ BNL	1	34	24	0	59
Iron (90 rad) @ BNL	0	33	0	0	33
Iron (160 rad) @ BNL	0	33	0	0	33
Iron (200 rad) @ BNL	0	0	0	0	0
Iron 16 + Proton 160	0	8	0	0	8
Proton (sham) @ LLU	5	45	0	19	69
Proton (50 rad) @ LLU	6	54	0	50	110
Proton (90 rad) @ LLU	0	53	0	60	113
Proton (160 rad) @ LLU	3	41	0	30	74
Proton (300 rad) @ LLU	0	39	0	0	39
Proton (500 rad) @ LLU	0	36	0	0	36
Proton (900 rad) @ LLU	0	11	0	0	11
Cobalt (sham) @ LLU	4	18	0	20	42
Cobalt (50 rad) @ LLU	2	20	0	57	79
Cobalt (90 rad) @ LLU	0	17	0	60	77
Cobalt (160 rad) @ LLU	3	19	0	32	54
Cobalt (300 rad) @ LLU	0	18	0	0	18
Cobalt (500 rad) @ LLU	1	16	0	0	17
Cobalt (900 rad) @ LLU	0	8	0	0	8
Cesium (sham) @ JHU	2	20	0	0	22
Cesium (50 rad) @ JHU	1	20	0	0	21
Cesium (90 rad) @ JHU	0	19	0	0	19
Cesium (160 rad) @ JHU	2	19	0	0	21
Cesium (300 rad) @ JHU	0	16	0	0	16
Cesium (500 rad) @ JHU	0	16	0	0	16
Cesium (900 rad) @ JHU	0	17	0	0	17
TOTAL	54	929	400	328	1711

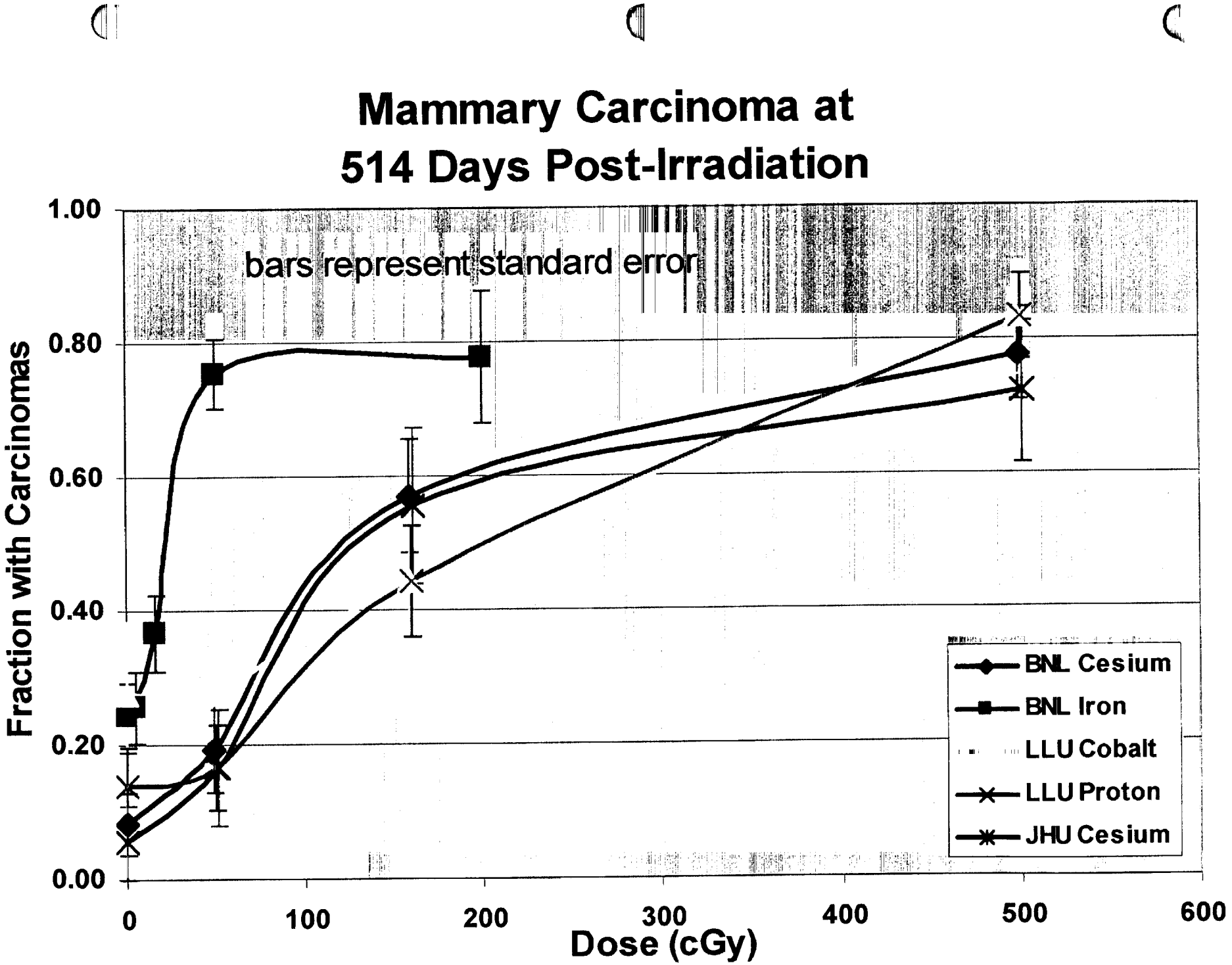
* For chemoprevention studies, approximately one-half of the May-99, Nov-99 and Apr-00 rats were administered Tamoxifen.

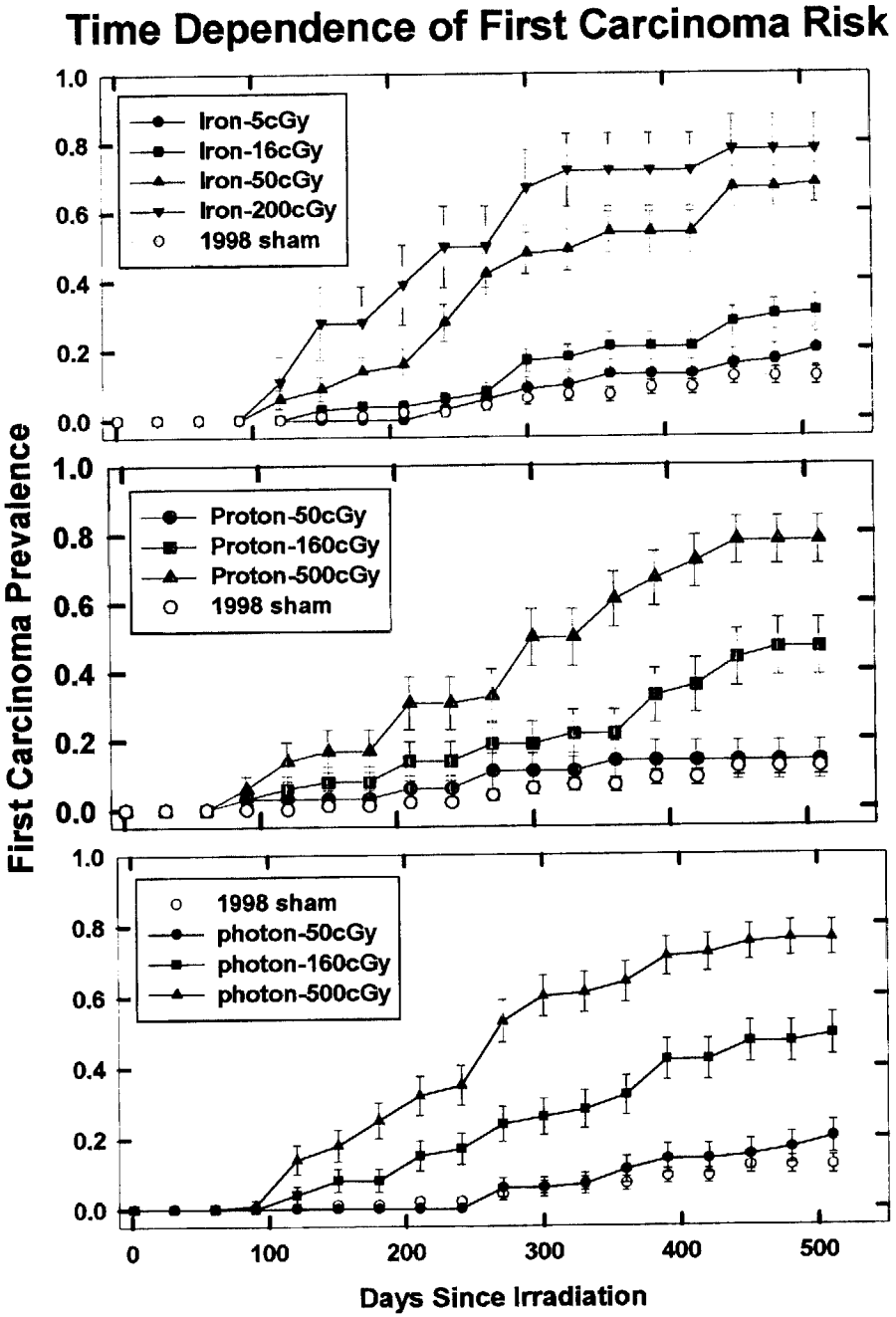
Suspected Primary Cause of Death (1998 Cohort)



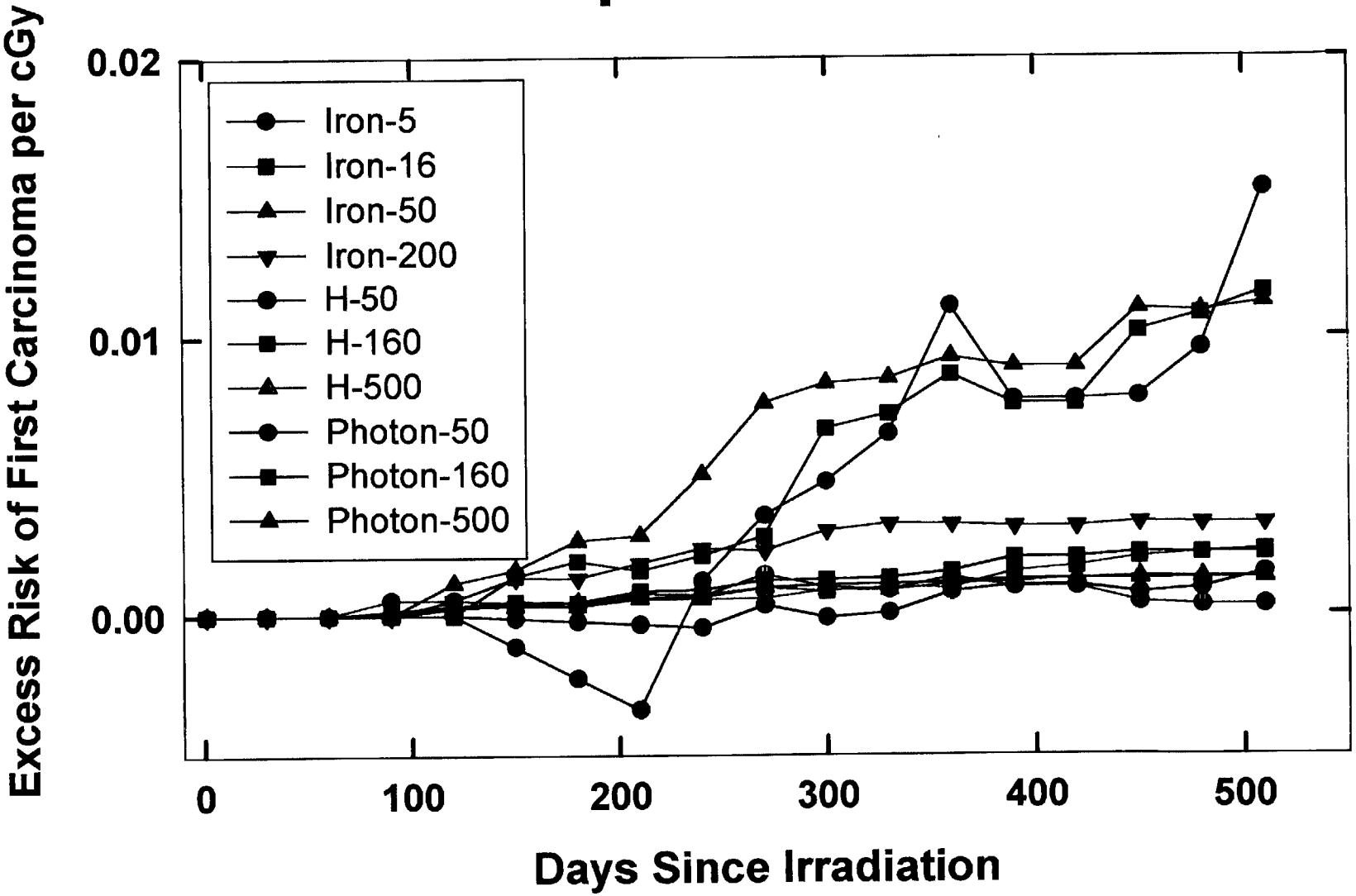
Total Resected Mammary Tumors at 514 Days Post-Irradiation



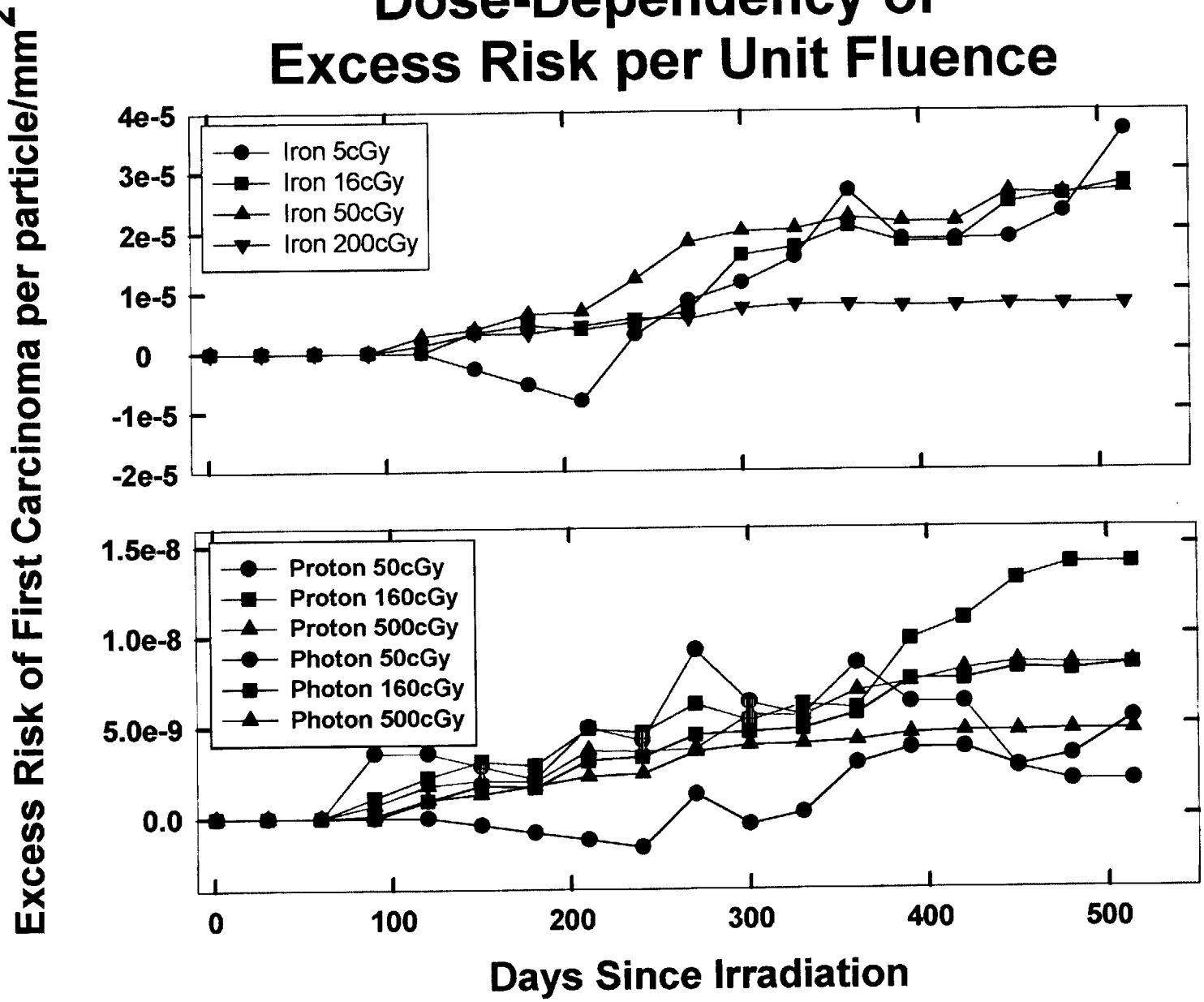

Mammary Carcinoma at
514 Days Post-Irradiation
bars represent standard error
Fraction with Carcinomas
1.00
0.80
0.60
0.40
0.20
0.00
0
100
200
300
400
500
600
Dose (cGy)
BNL Cesium
BNL Iron
LLU Cobalt
LLU Proton
JHU Cesium


Time Dependence of First Carcinoma Risk
First Carcinoma Prevalence
Iron-5cGy
Iron-16cGy
Iron-50cGy
Iron-200cGy
1998 sham
Proton-50cGy
Proton-160cGy
Proton-500cGy
1998 sham
1998 sham
photon-50cGy
photon-160cGy
photon-500cGy
0.0
0.2
0.4
0.6
0.8
1.0
0
100
200
300
400
500
Days Since Irradiation

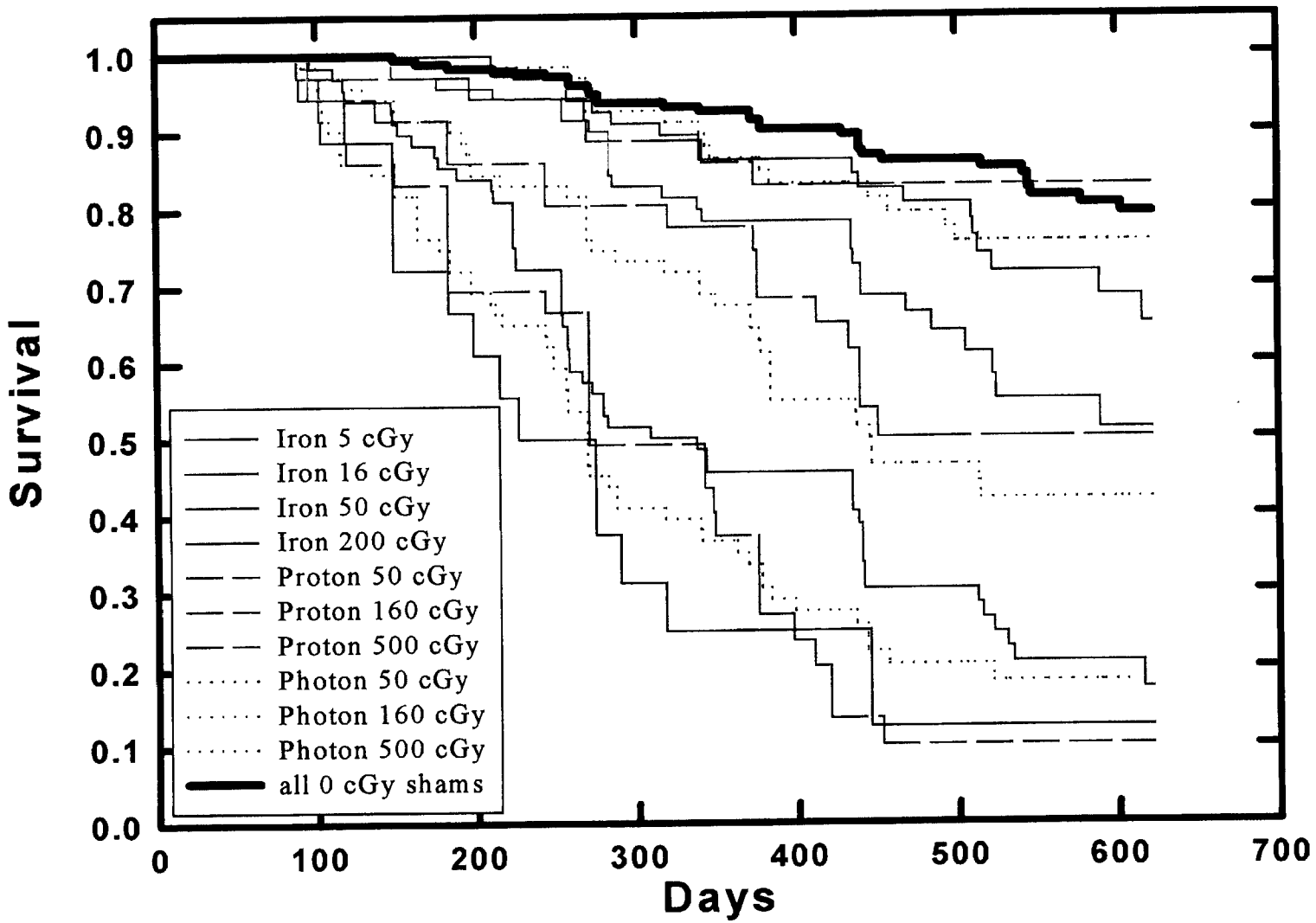
Dose Dependency of Excess Risk per Unit Dose



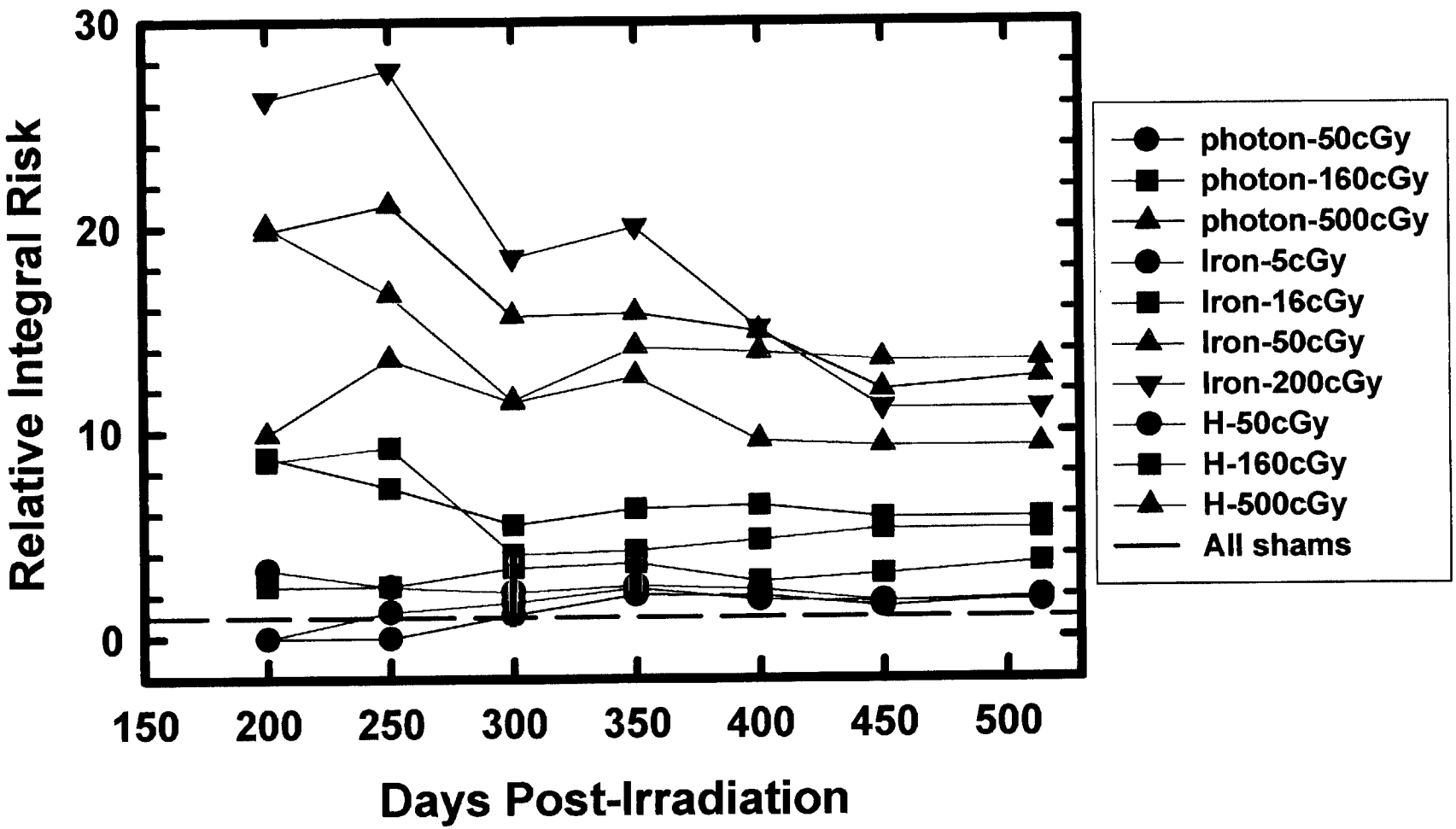
Dose-Dependency of Excess Risk per Unit Fluence



Survival Without a Mammary Carcinoma
1998 Cohort



Relative Integral Risk of First Carcinoma
1998 Cohort

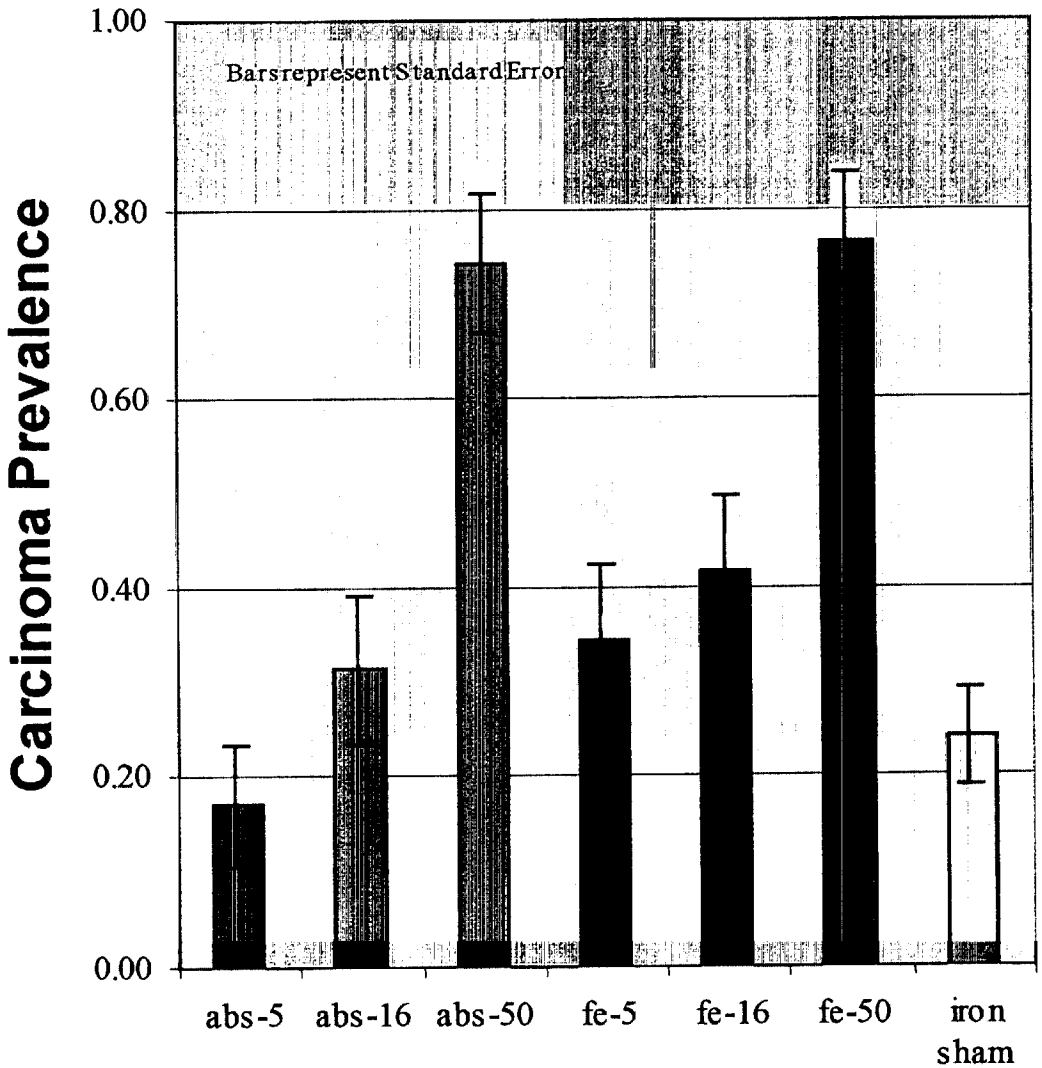








Iron Ions with or without Lucite Absorber

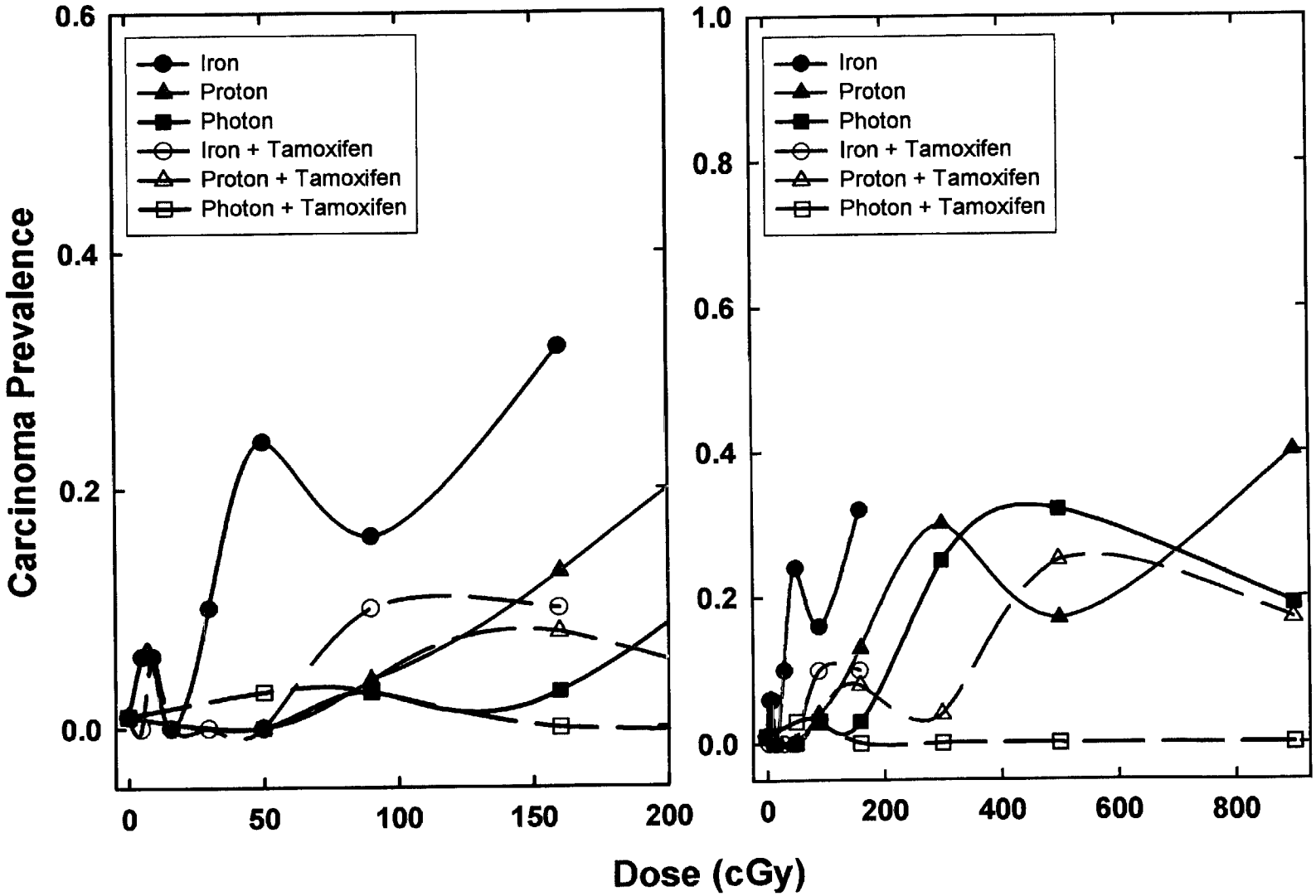




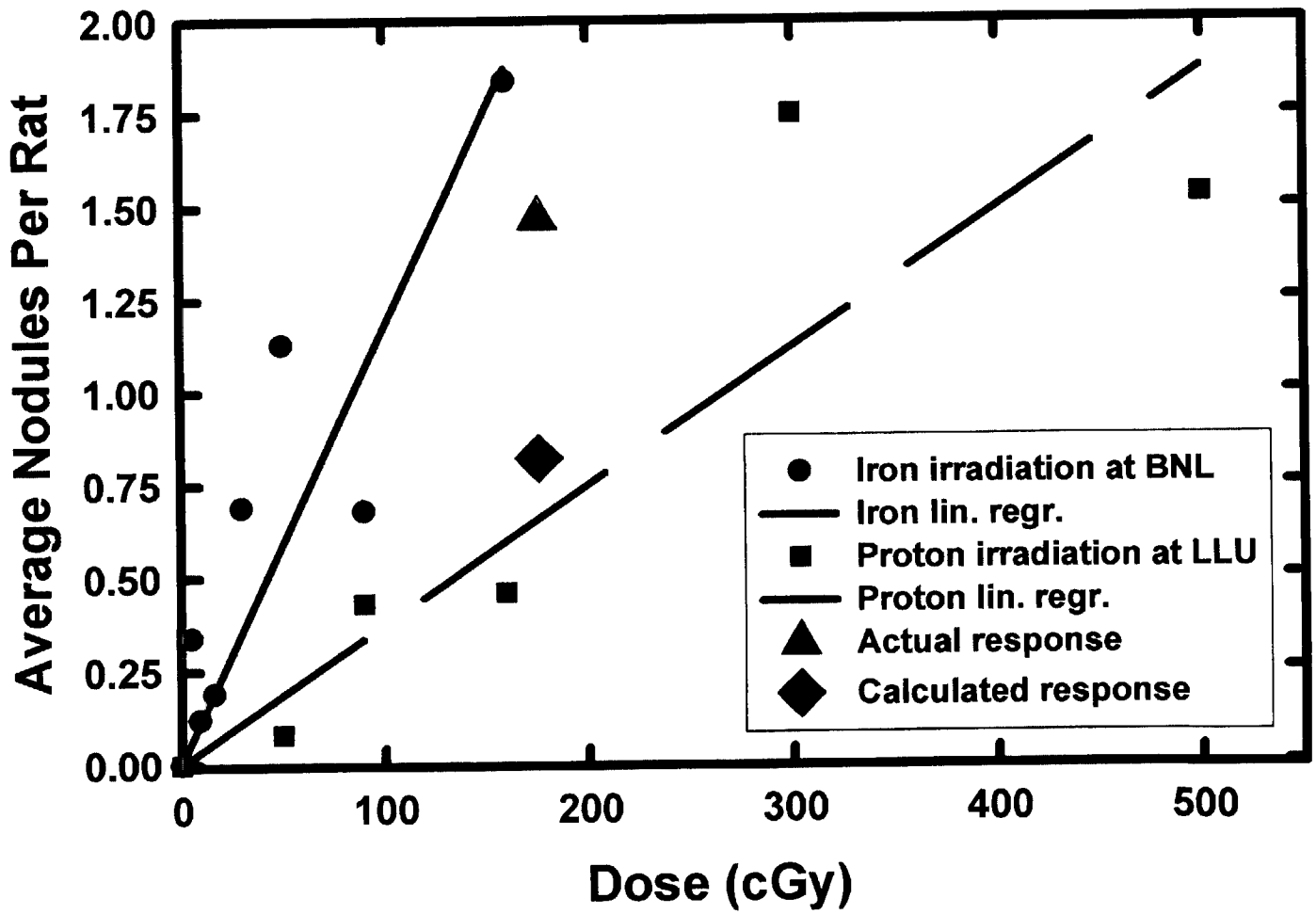


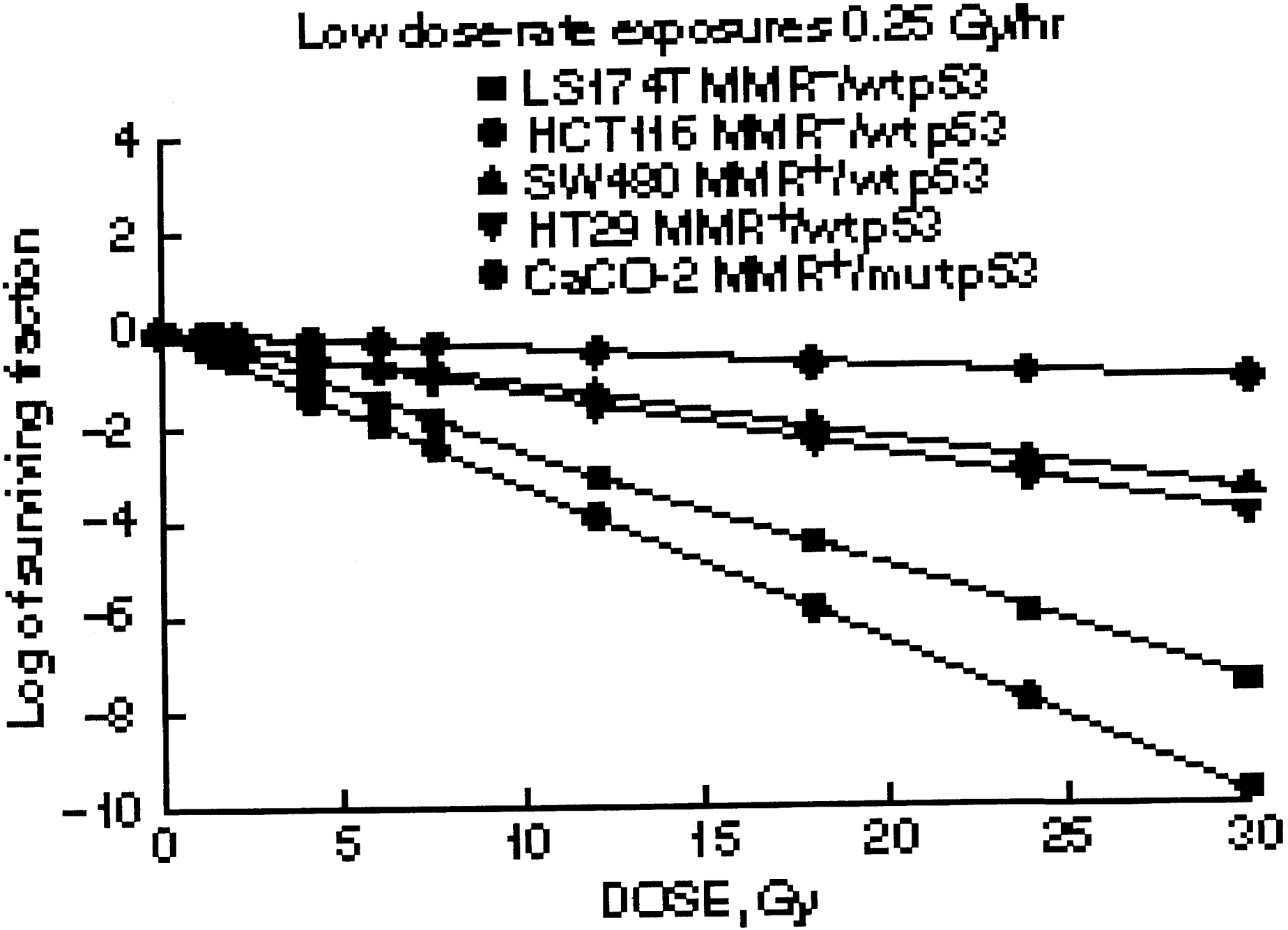


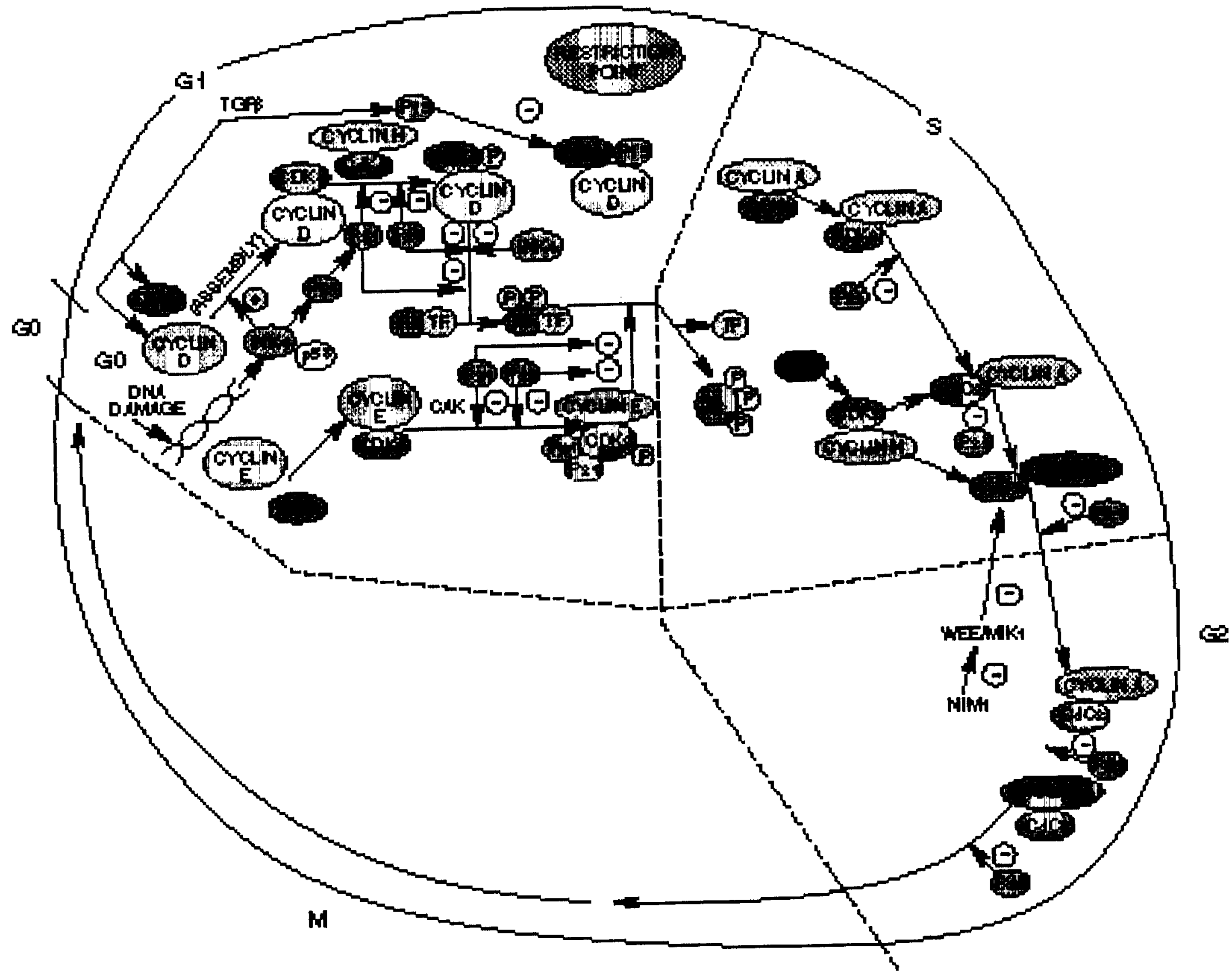
Carcinoma Prevalence at 378 Days Post-Irradiation



Nodule Response Versus Dose
1999 Spring Cohort (One Year Post-irrad)




Low dose-rate exposures 0.25 Gy/hr
LS174T MMR-/wtp53
HCT116 MMR-/wtp53
SW480 MMR+/wtp53
HT29 MMR+/wtp53
CaCO-2 MMR+/mutp53
Log of surviving fraction
4
2
0
-2
-4
-6
-8
-10
0
5
10
15
20
25
30
DOSE, Gy


G1
S
G2
M
G0
G0
TGFβ
CYCLIN H
CYCLIN D
CYCLIN D
CYCLIN D
CYCLIN D
ASSEMBLY
p53
DNA
DAMAGE
CYCLIN E
CYCLIN E
CAK
TF
TF
TF
CYCLIN A
CYCLIN A
CYCLIN A
CYCLIN B
WEE/MIK1
NIM1

$$d[Cyc_i]/dt = v_{Si}[TF_i] - v_{Di}[Cyc_i] - \Sigma_j k_{ij}[Cyc_i][cdk_j]$$

$$d[Cyc^P{}_i]/dt = \Sigma_j v_{Dij}[M^P{}_{ij}] - v^P{}_{Di}[Cyc^P{}_i]$$

$$d[cdk_j]/dt = -\Sigma_i k_{ij}[Cyc_i][cdk_j] + \Sigma_i v_{Dij}[M^P{}_{ij}] - \Sigma_\alpha k_{j\alpha}[cdk_j][Ink_\alpha] + \Sigma_\alpha v_{D\alpha}[Ink_\alpha\text{-}cdk_j]$$

$$d[M^I{}_{ij}]/dt = k_{ij}[Cyc_i][cdk_j]\delta_{I0} - v_{Dij}[M^I{}_{ij}] - \Sigma_J \varepsilon_J k^{P,Q}{}_{ij}[M^J{}_{ij}] + \Sigma_J \varepsilon_J k^{Q,P}{}_{ij}[M^J{}_{ij}]$$

$$- \Sigma_\beta k_{ij\beta}[KI_\beta][M^I{}_{ij}]\delta_{I0} + \Sigma_\beta v_{D\beta}[KI_\beta\text{-}M^0{}_{ij}]\delta_{I0}$$

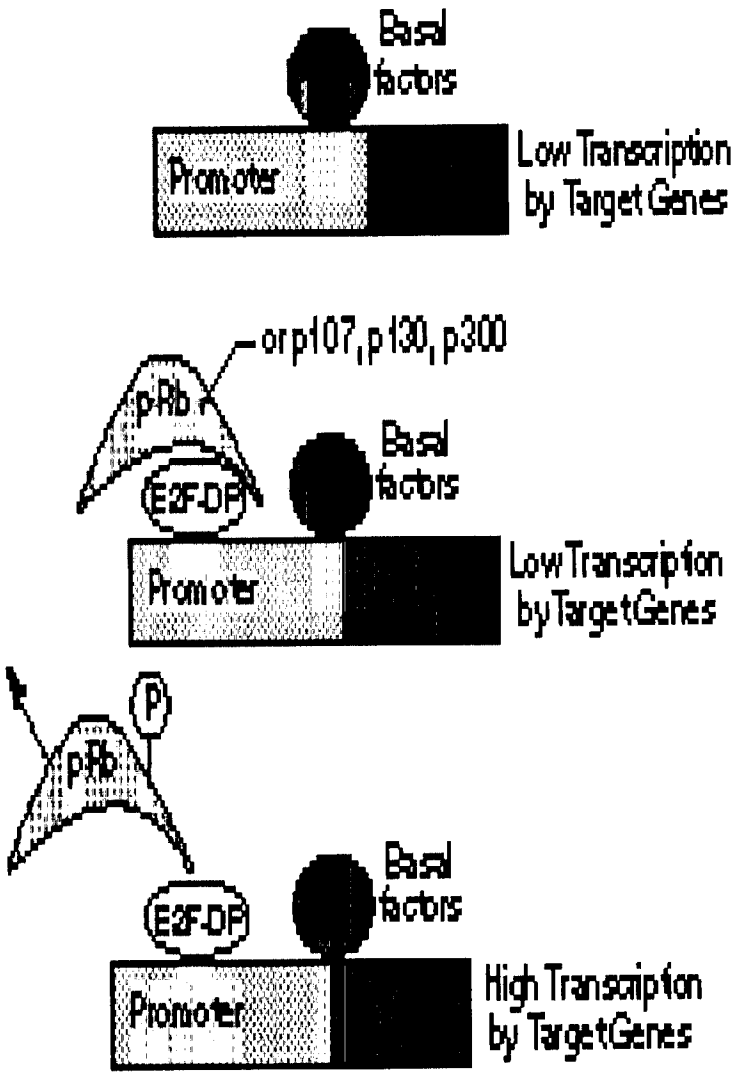
$$d[pRb^P]/dt = \Sigma_j r^P{}_{ij}[M^P{}_{ij}][pRb\text{-}E2F_{i+1}]/(K+[pRb\text{-}E2F_{i+1}]) - r^Q{}_{G2/M}[M^P{}_{G2/M}][pRb^P]$$

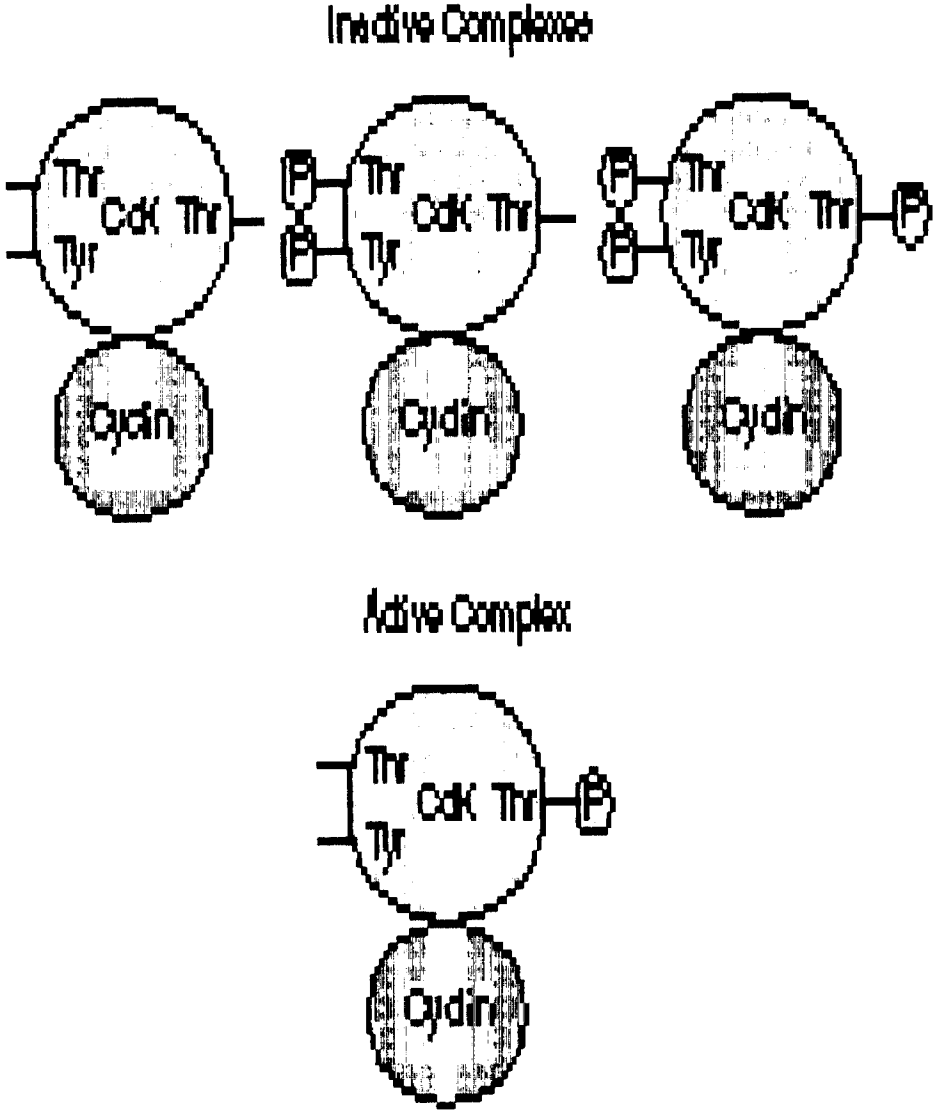
$$d[E2F_{i+1}]/dt = \Sigma_j r^P{}_{ij}[M^P{}_{ij}][pRb\text{-}E2F_{i+1}]/(K+[pRb\text{-}E2F_{i+1}]) - k_{F\ i+1}[E2F_{i+1}][pRb^0]$$

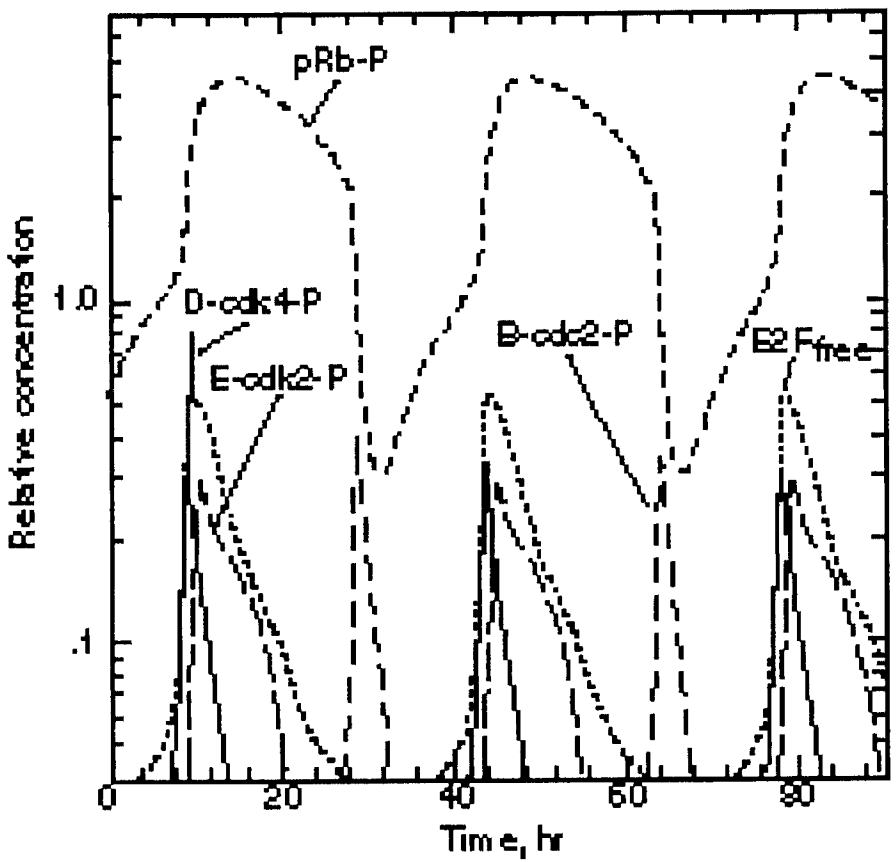
$$d[Ink_\alpha]/dt = v_{S\alpha}[TF_\alpha] - v_{D\alpha}[Ink_\alpha] - \Sigma_j k_{j\alpha}[cdk_j][Ink_\alpha]$$

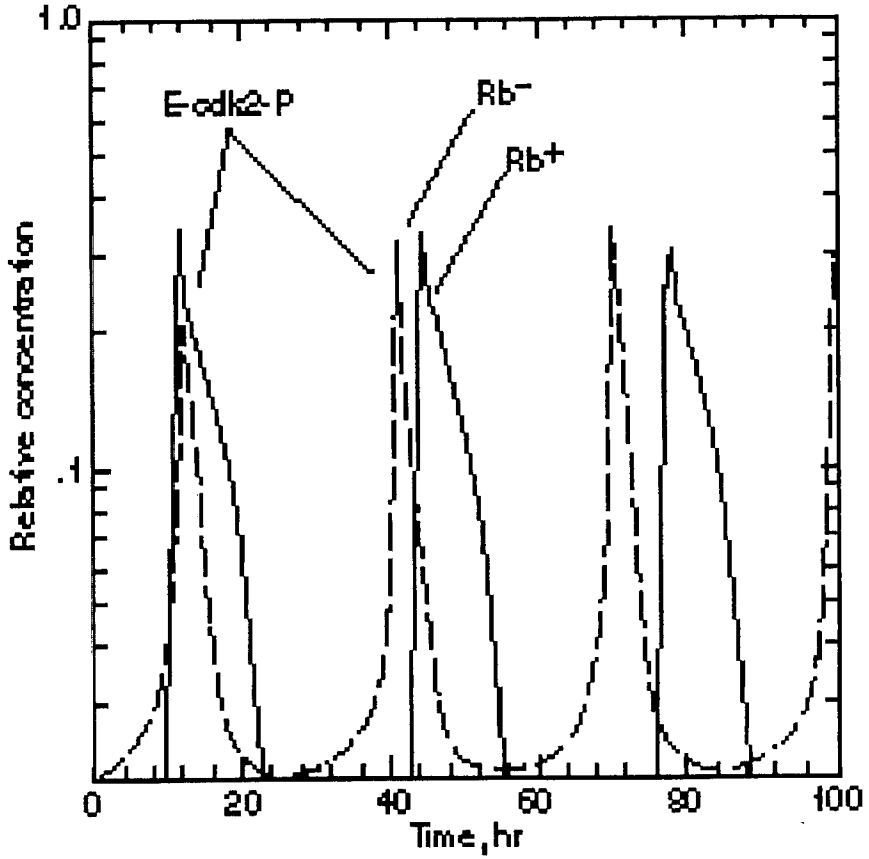
$$d[Ink_\alpha\text{-}cdk_j]/dt = k_{j\alpha}[cdk_j][Ink_\alpha] - v_{D\alpha}[Ink_\alpha\text{-}cdk_j]$$

$$d[KI_\beta]/dt = v_{S\beta}[TF_\beta] - v_{D\beta}[KI_\beta] - \Sigma_{ij} k_{ij\beta}[KI_\beta][M^0{}_{ij}]$$


Basal factors
Promoter
Low Transcription by Target Genes
or p107, p130, p300
p-Rb
E2F-DP
Basal factors
Promoter
Low Transcription by Target Genes
P
p-Rb
E2F-DP
Basal factors
Promoter
High Transcription by Target Genes


Inactive Complexes
Thr
Tyr
CdK
Thr
Cyclin
P
Active Complex


pRb-P
D-cdk4-P
E-cdk2-P
B-cdc2-P
E2F free
Relative concentration
1.0
.1
0
20
40
60
80
Time, hr


E-cdk2-P
Rb⁻
Rb+
Relative concentration
1.0
.1
0
20
40
60
80
100
Time, hr

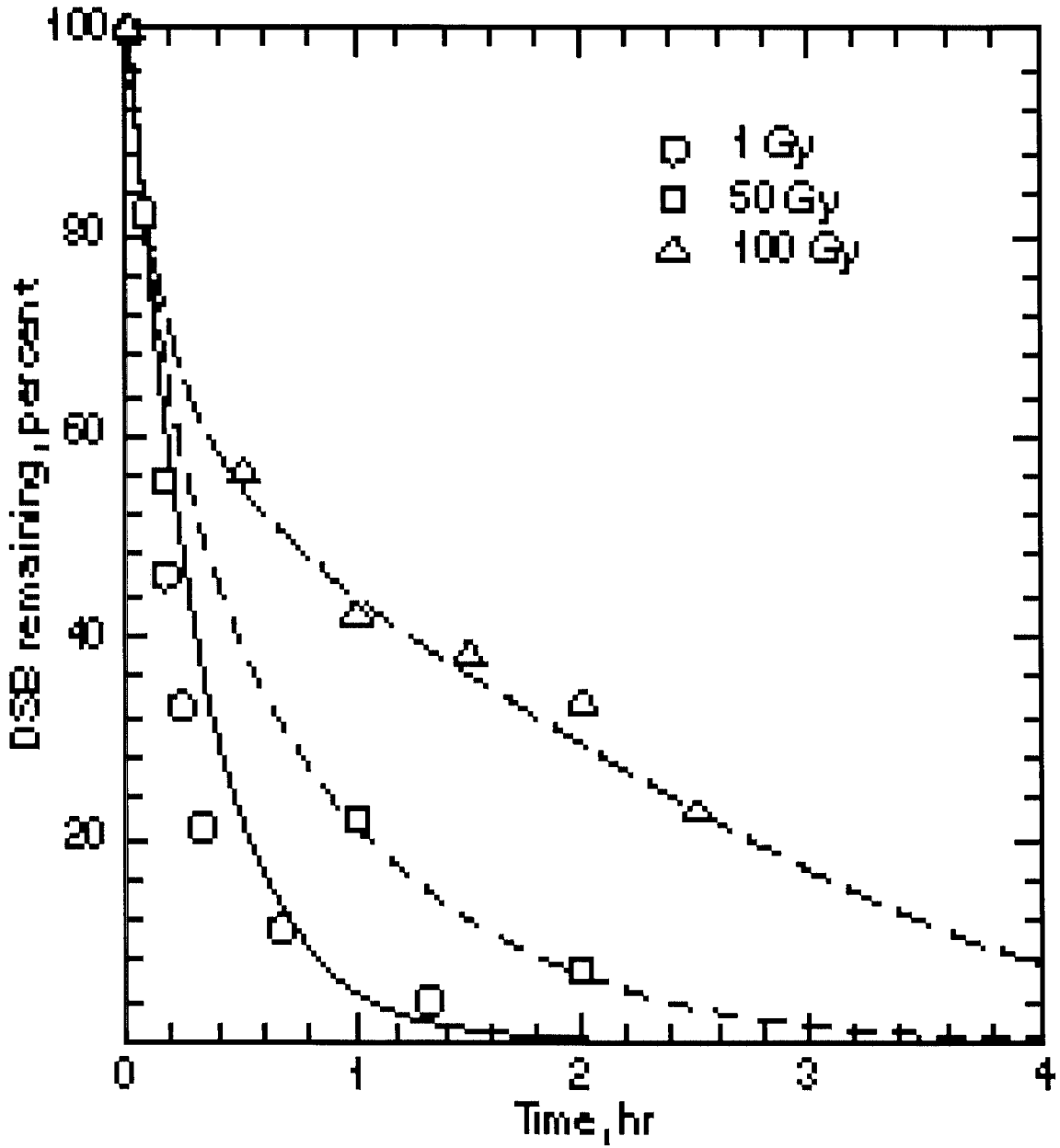
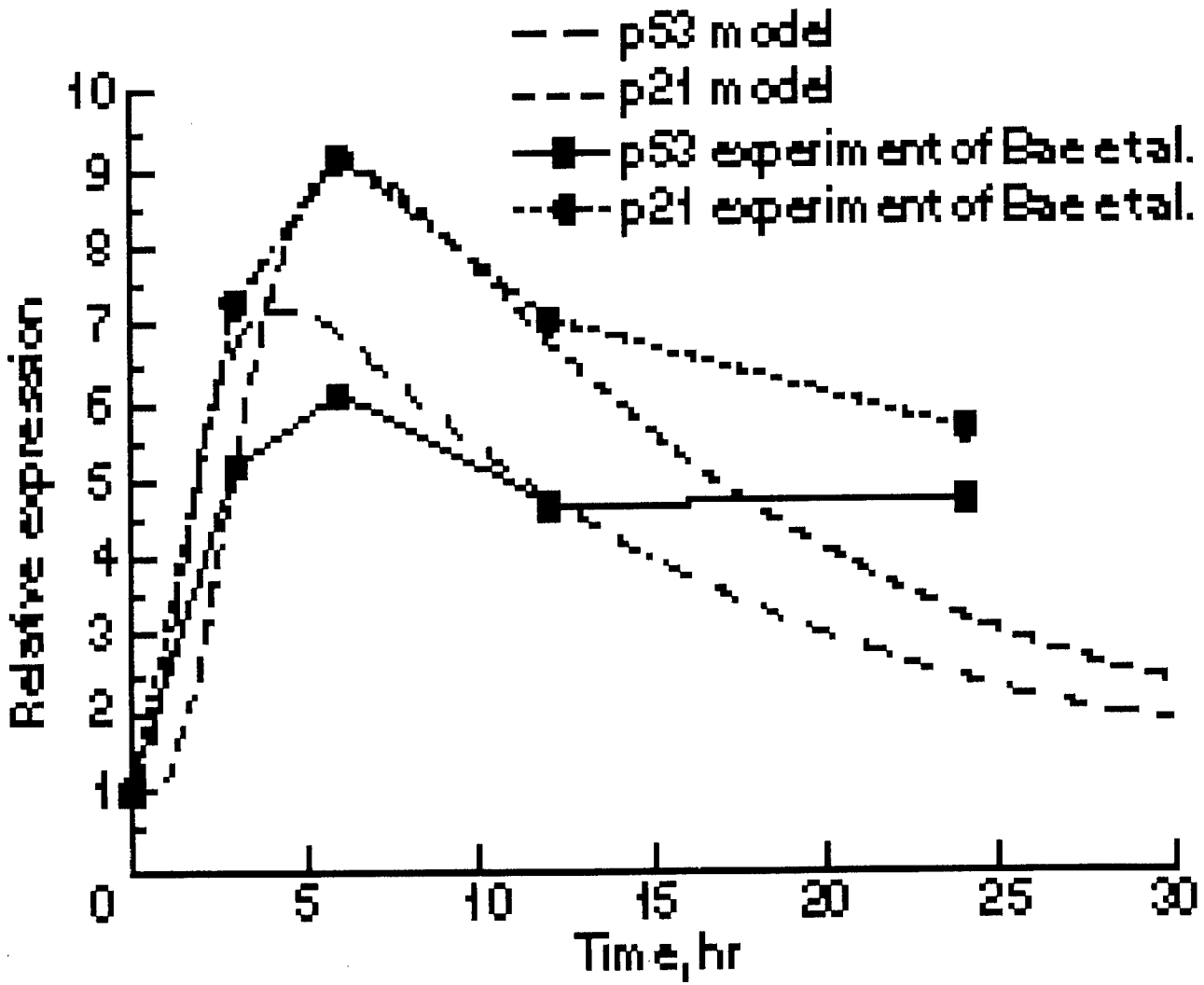
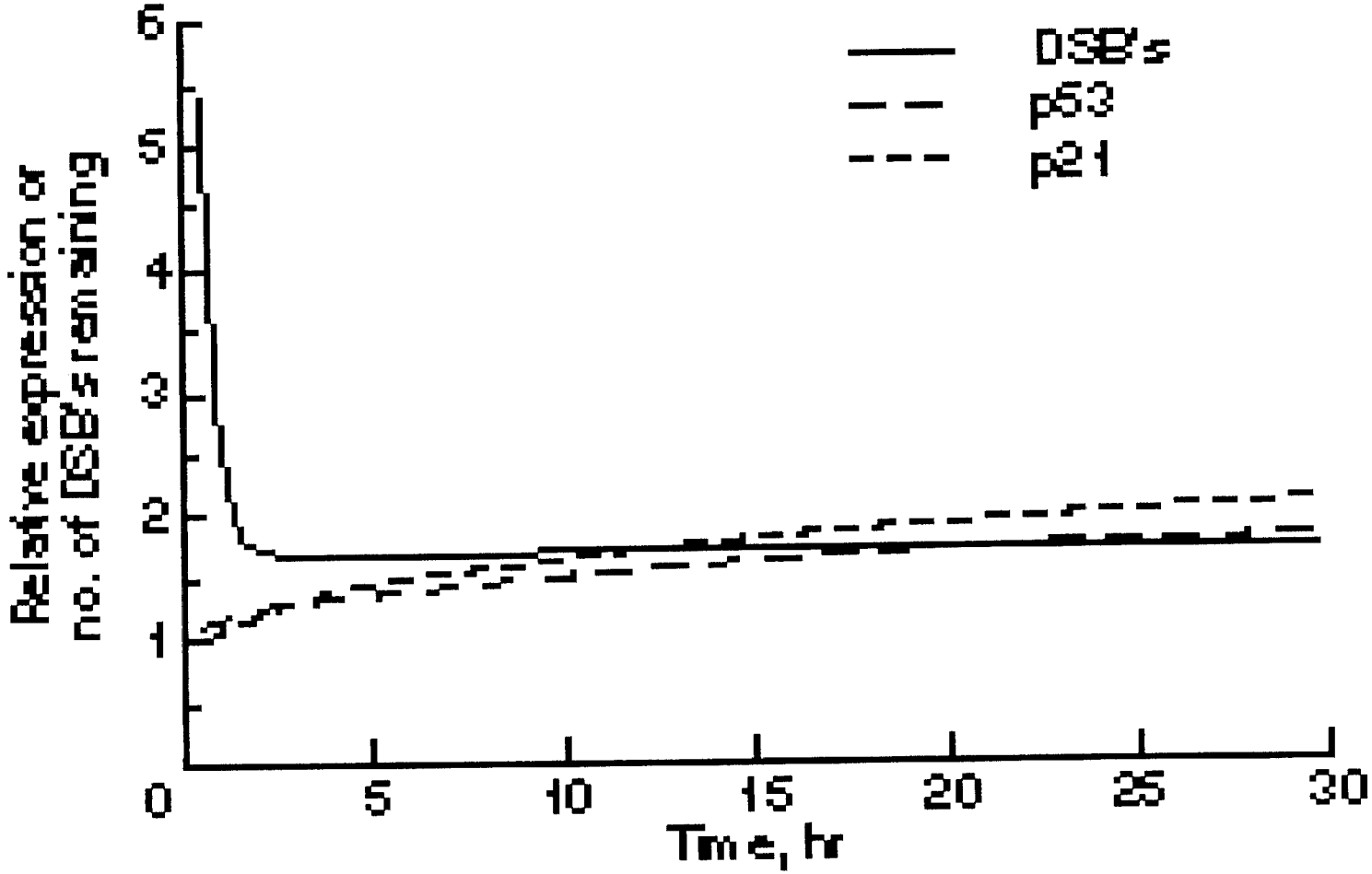


Fig. 17. Calculations of DSB repair versus time after exposure. Also shown are DSB repair data of McWilliams et al., 1983, and G. P. Van Der Schans et al., 1983.


p53 model
p21 model
p53 experiment of Bae et al.
p21 experiment of Bae et al.
Relative expression
Time, hr
0
1
2
3
4
5
6
7
8
9
10
5
10
15
20
25
30


DSB's
p53
p21
Relative expression or no. of DSB's remaining
0
1
2
3
4
5
6
0
5
10
15
20
25
30
Time, hr

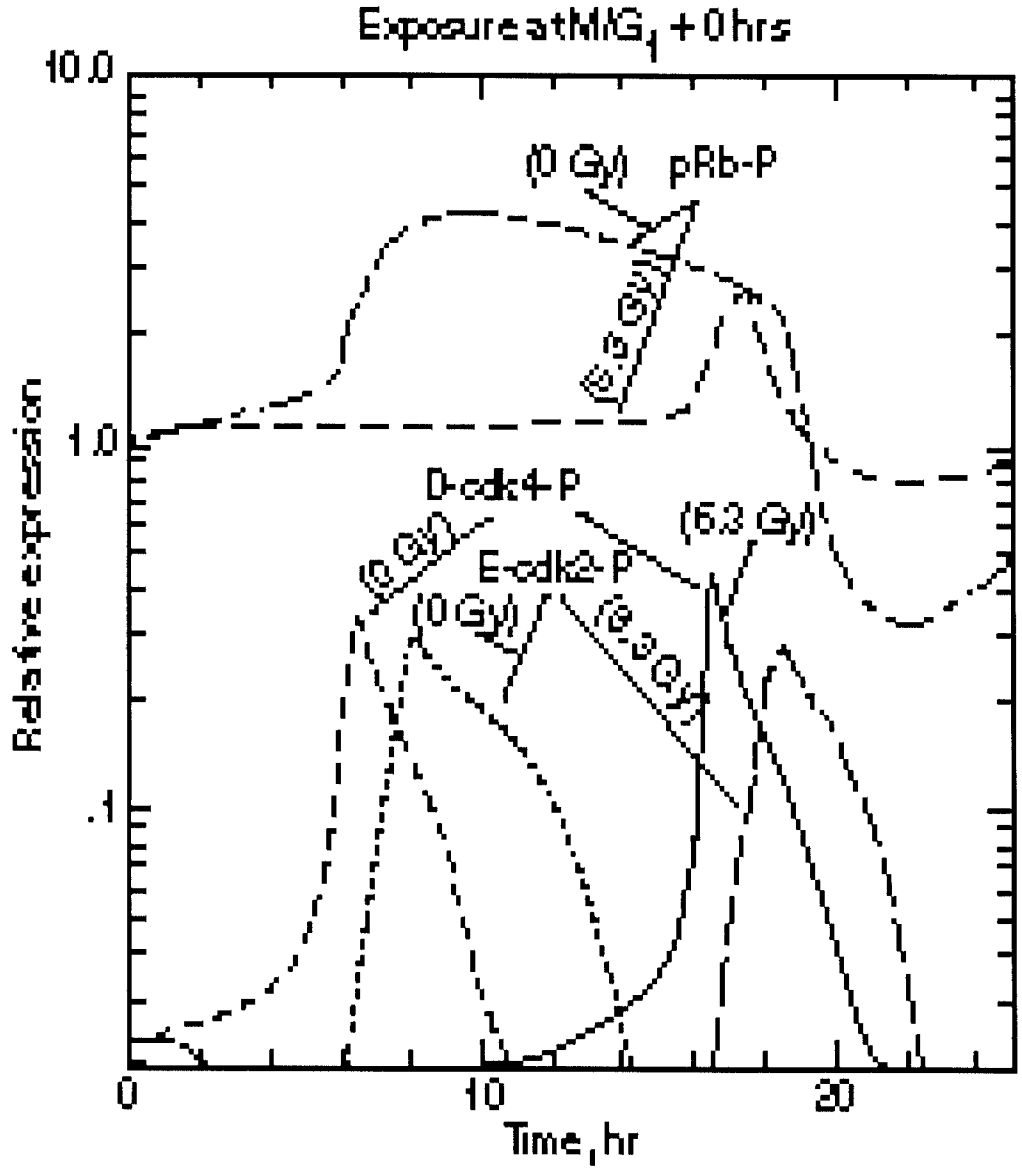
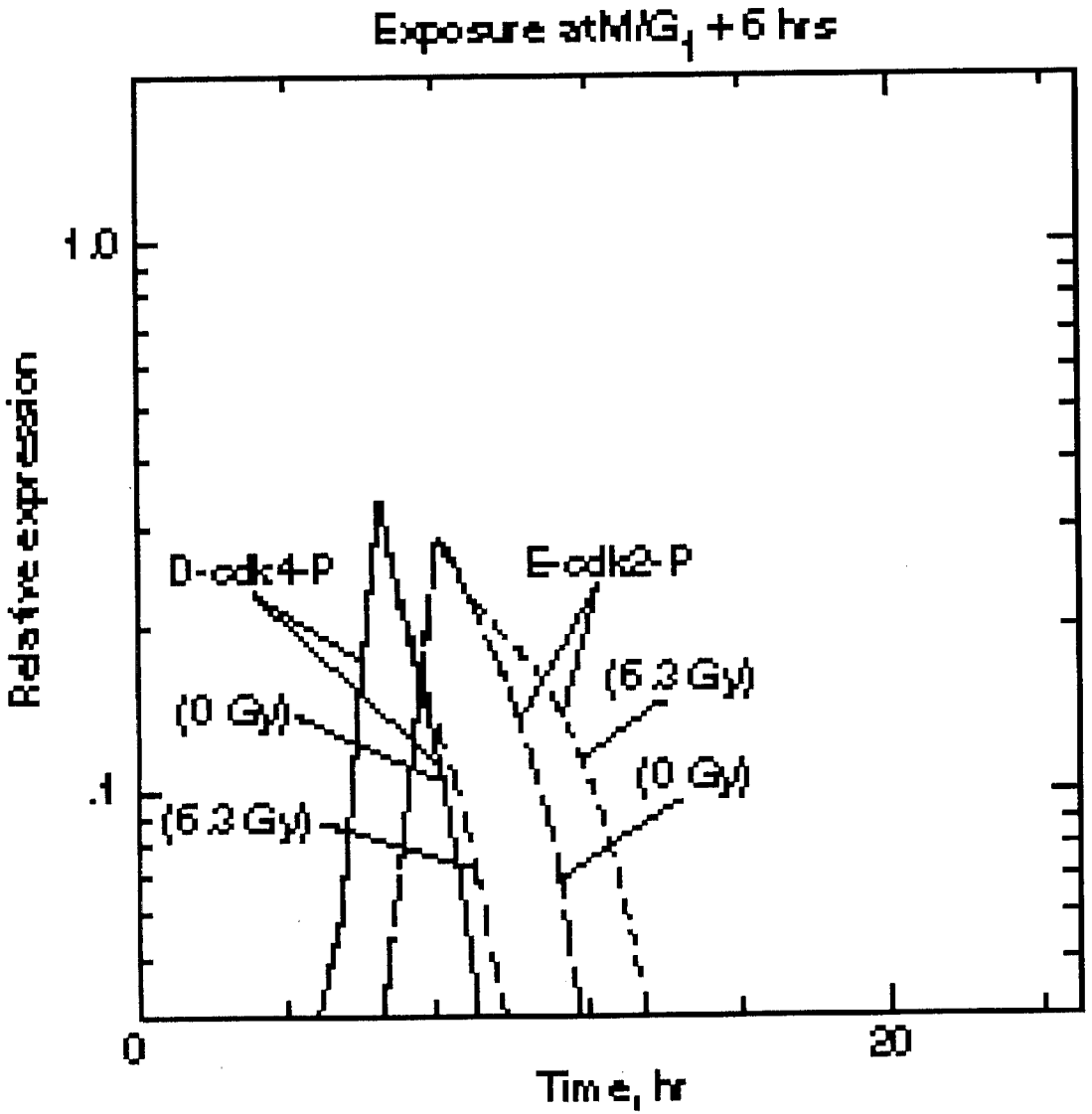
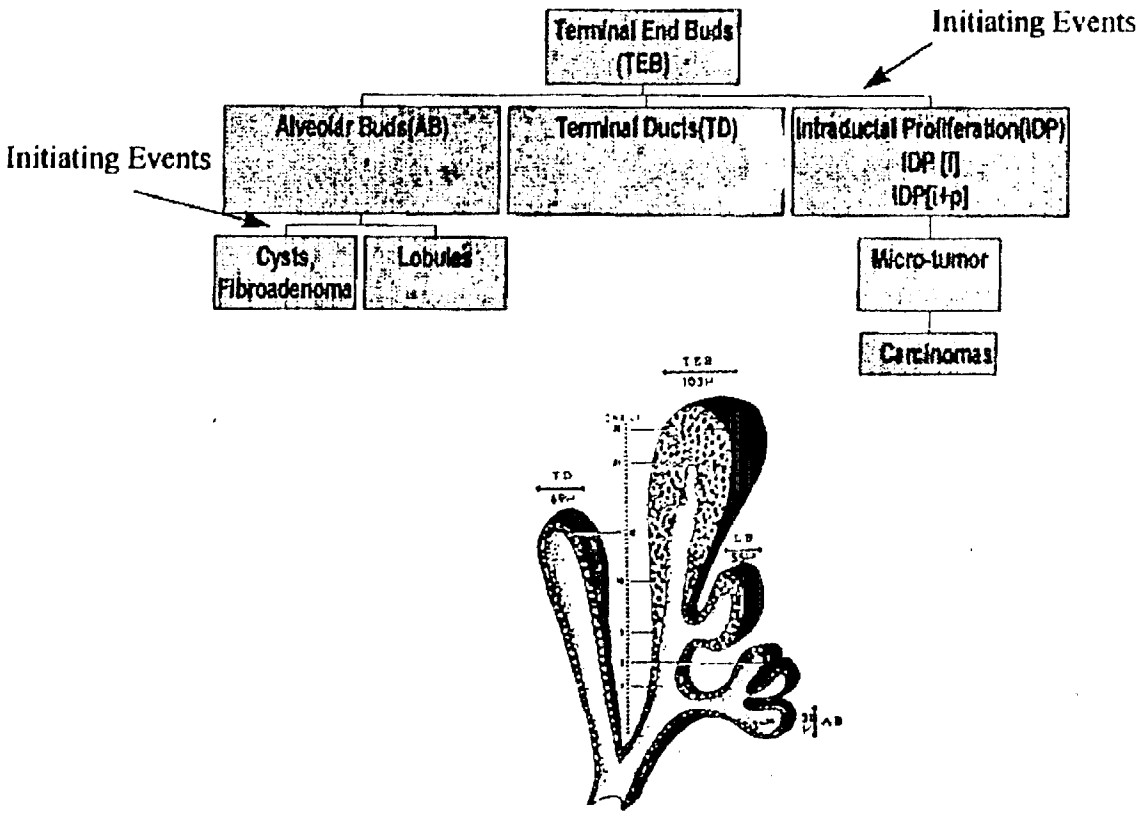


Fig. 20a. Calculations of relative expression of several proteins after acute exposure of 6.3 Gy at M/G1 border.


Exposure at M/G1 + 6 hrs
1.0
.1
0
20
Relative expression
Time, hr
D-cdk4-P
E-cdk2-P
(6.3 Gy)
(0 Gy)
(0 Gy)
(6.3 Gy)

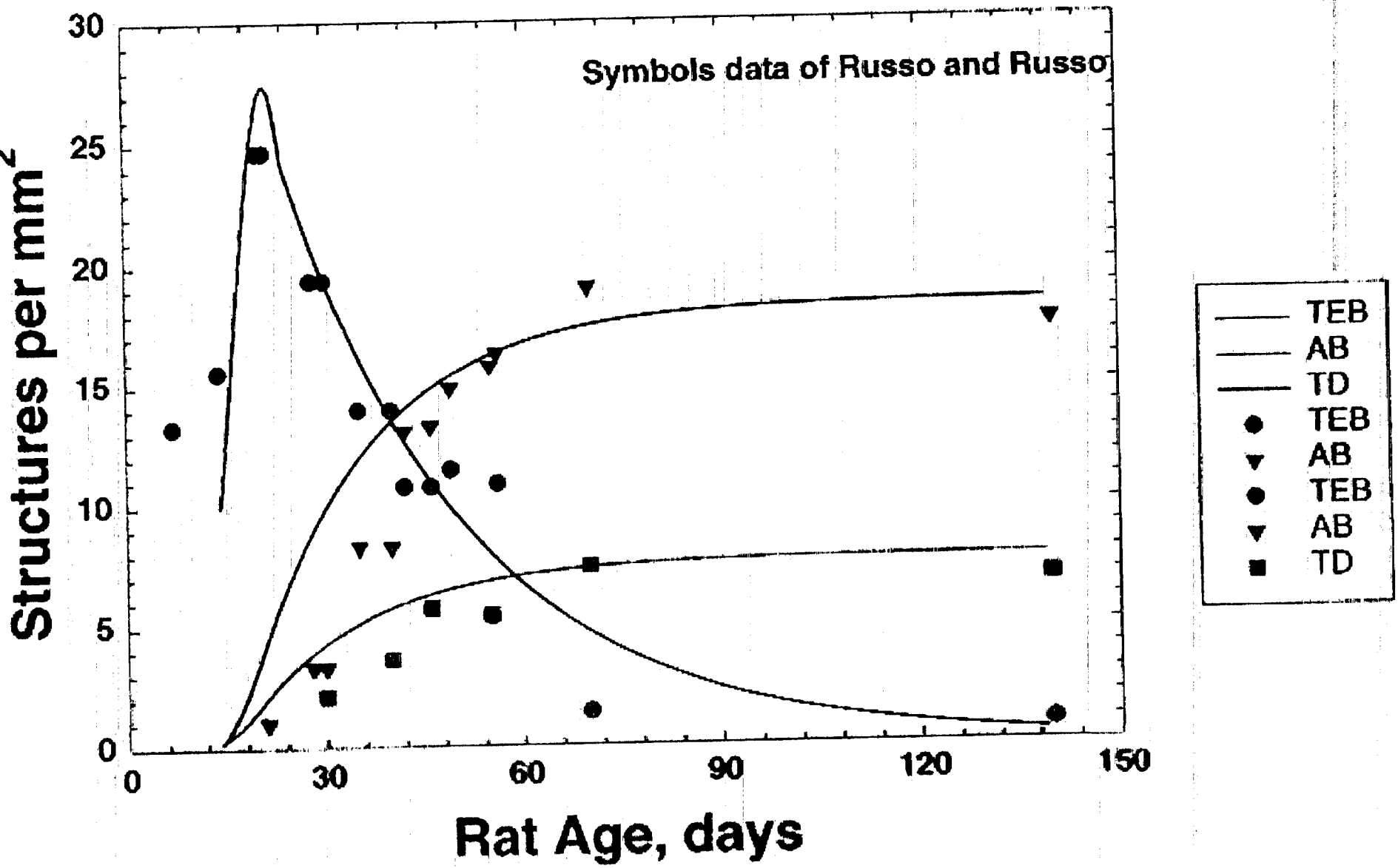
Model of Differentiation in the Rat Mammary Gland

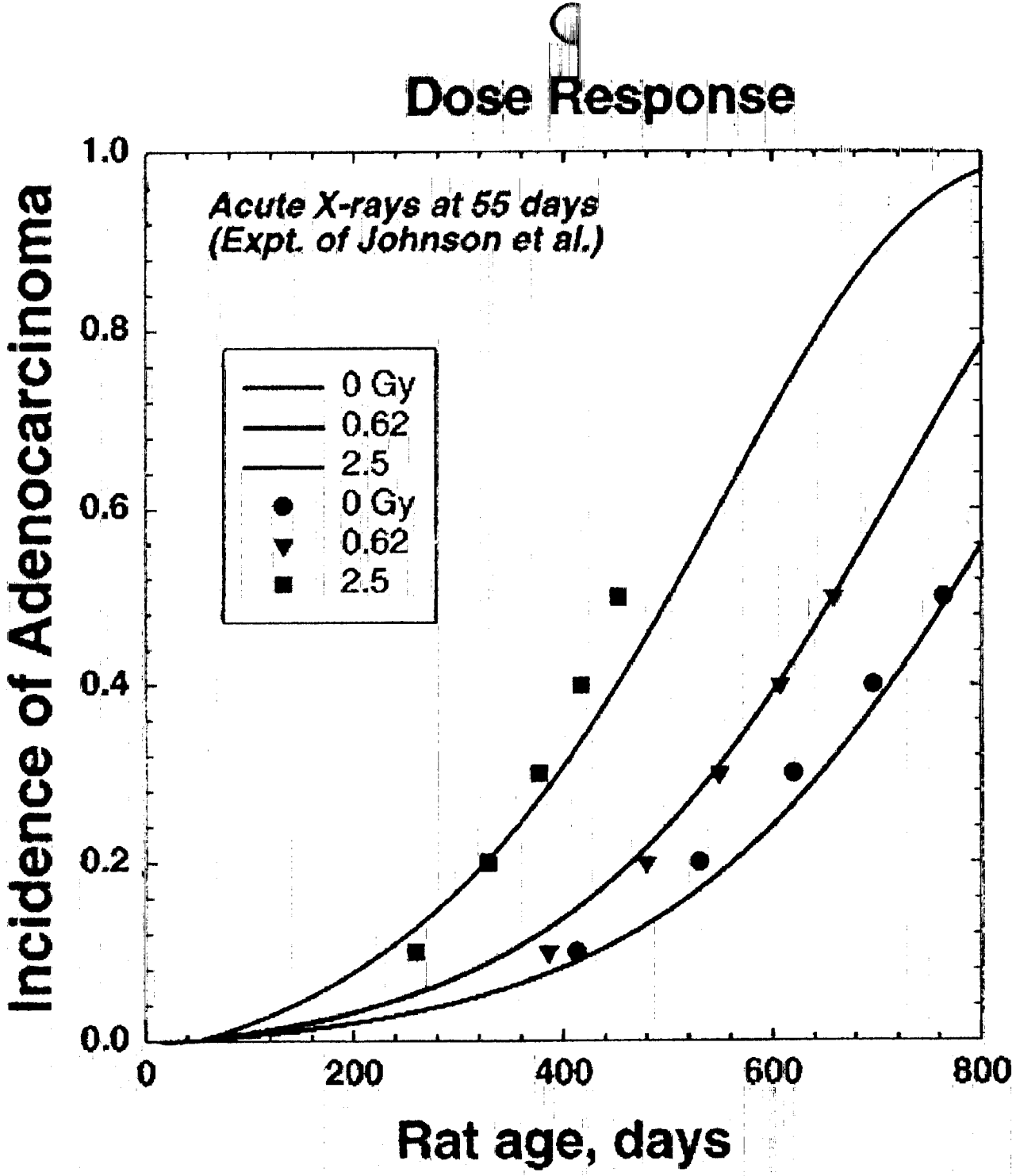


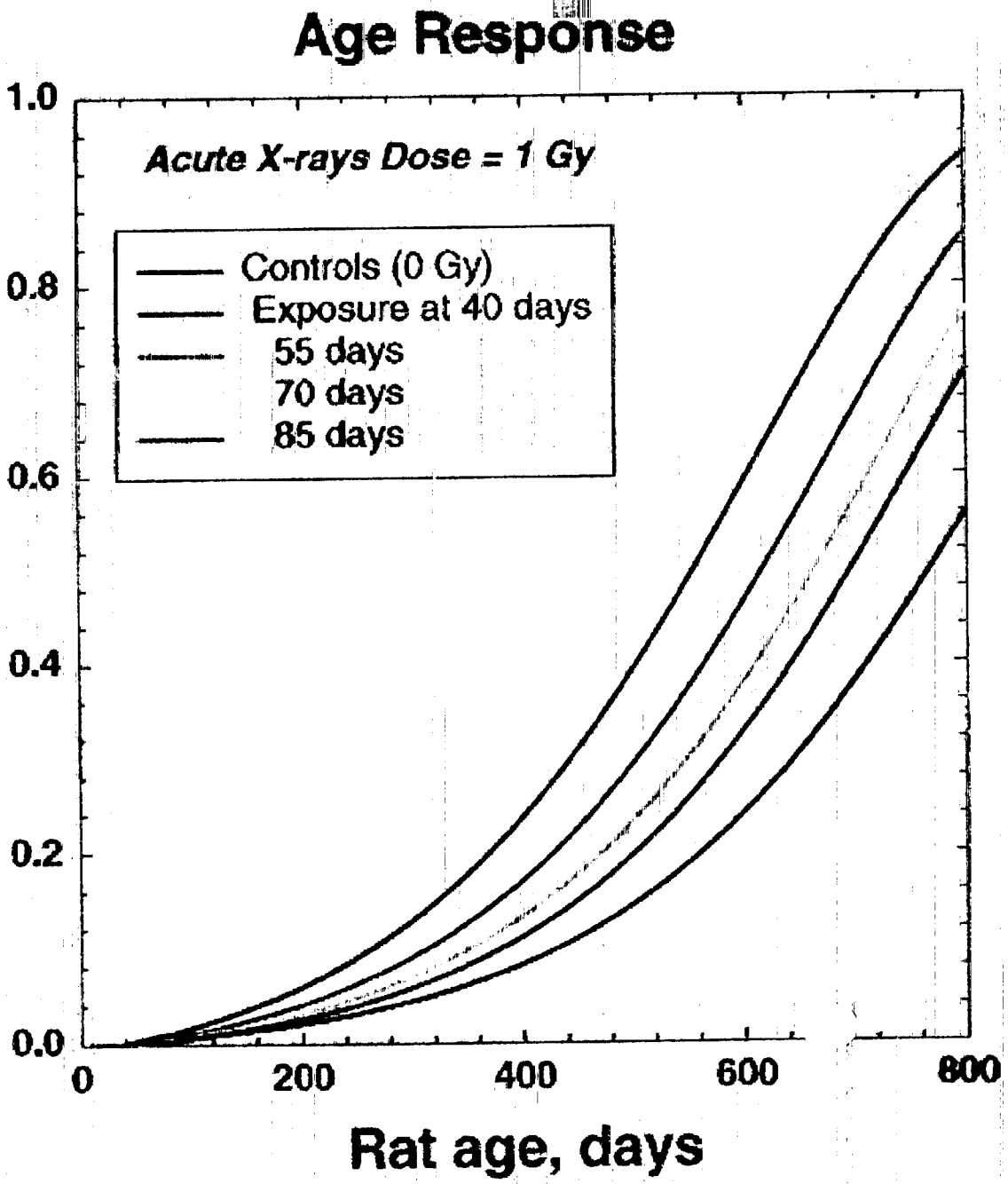
Protein	[TFi]	v_s, model	v_d, model	v_d, expt.	k^p_o
[Cyclin D]					
-TEB	mitogens	0.5,hr^{-1}	½, hr^{-1}	½, hr^{-1}	0.03, hr^{-1}
-TD	mitogens	0.15	½	½	0.03
-AB	mitogens	0.1	½	½	0.03
[Cyclin E]	[E2Fe]	1.08	1/3	-	0.015
[Cyclin A}	[E2Fa]	0.38	1/4	1/4	0.015
[Cyclin B]	[E2Fb]	0.53	0.075	0.067	0.09
[p16]	[TF_{16}]	1.1	0.125	<1/3	-

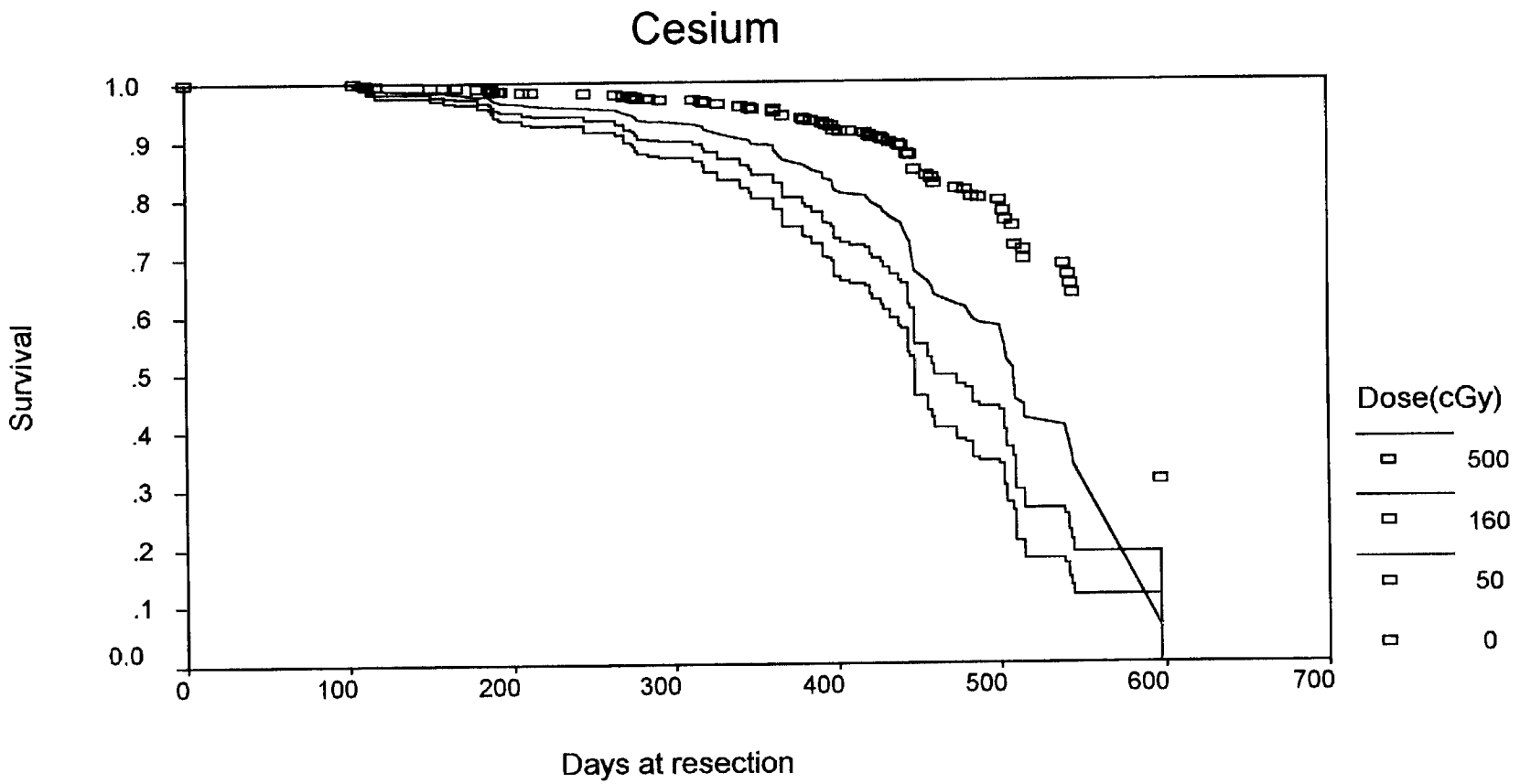
Group	Structure	Gl, hr.	S, hr.	G2, hr	M, hr.	T_{cycle},hr	Growth Fraction
Young Virgin	TEB	.72+/.42	7.2+/.80	1.08+/.11	1.2+/.2	9.93+/.31	0.55
	TD	7.5+/.12	7.5+/.83	1.00+/.01	1.4+/.4	17.33+/.42	0.39
	AB	17.3+/1.1	8.7+/.61	1.00+/-.01	1.2+/.2	28.2+/1.98	0.13
Old Virgin	TEB	5.2+/.99	7.6+/.40	4.40+/-.40	1.6+/.2	18.8+/0.99	0.077
	TD	7.1+/.08	7.6+/.40	4.40+/-.40	1.4+/.2	20.57+/1.8	0.020
	AB	17.3+/1.4	8.2+/.72	3.60+/-.40	1.7+/.1	30.75+/-2.7	0.008

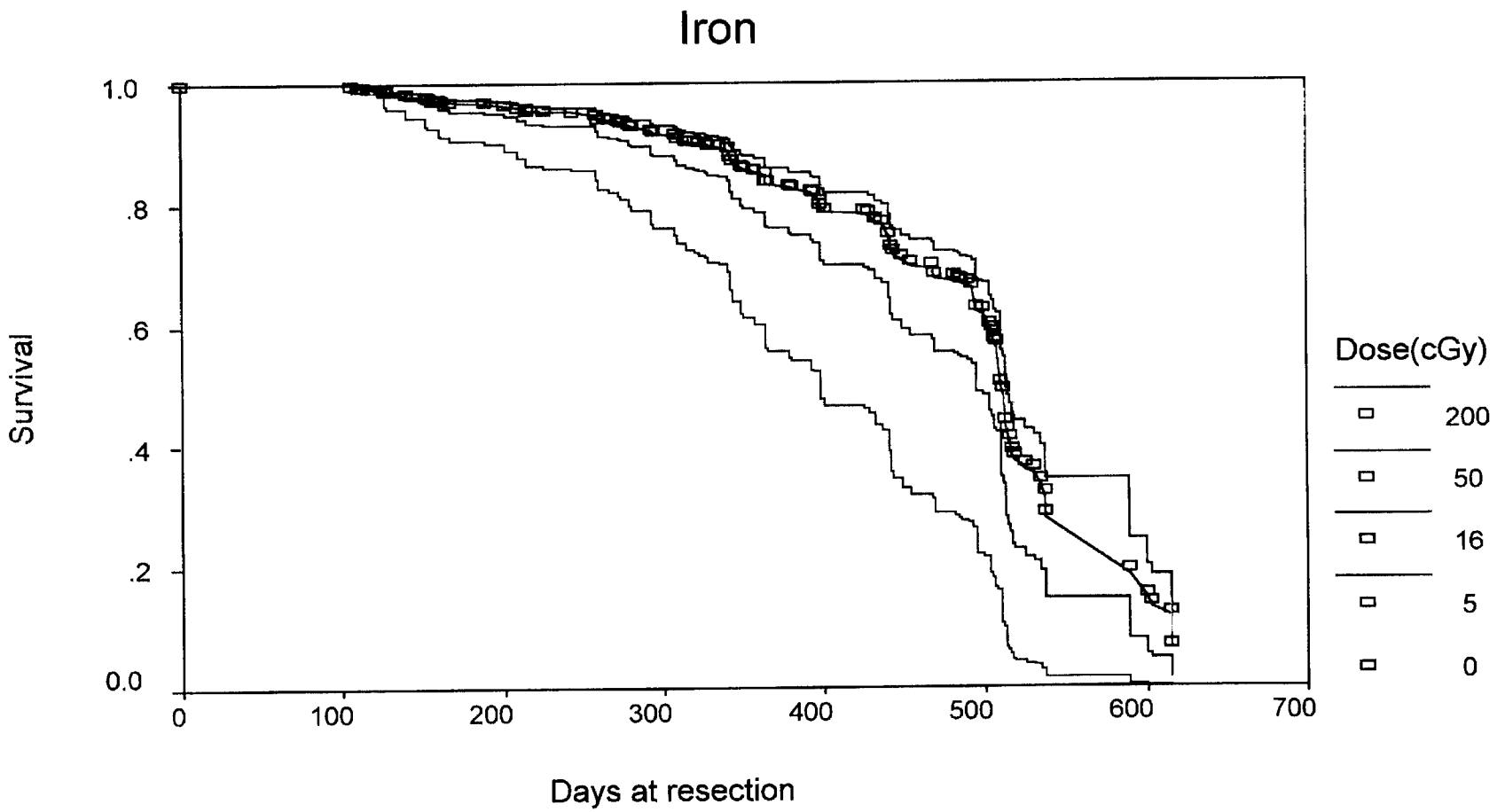
Mammary Gland Structures




Dose Response
Acute X-rays at 55 days
(Expt. of Johnson et al.)
0 Gy
0.62
2.5
0 Gy
0.62
2.5
Incidence of Adenocarcinoma
Rat age, days
0.0
0.2
0.4
0.6
0.8
1.0
0
200
400
600
800


Age Response
Acute X-rays Dose = 1 Gy
Controls (0 Gy)
Exposure at 40 days
55 days
70 days
85 days
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
Rat age, days


Cesium
Survival
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0.0
0
100
200
300
400
500
600
700
Days at resection
Dose(cGy)
500
160
50
0


Iron
Survival
1.0
.8
.6
.4
.2
0.0
0
100
200
300
400
500
600
700
Days at resection
Dose(cGy)
200
50
16
5
0







Chemoprevention of Radiation-Induced Rat Mammary Neoplasms

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EXECUTIVE SUMMARY

Chemoprevention is a pharmaceutical approach to arresting or reversing the process of carcinogenesis during cancer's typically prolonged latent period (often 20 years or more) before invasion or metastasis occurs. Surging scientific and public interest in applying chemoprevention strategies to people in the general population that have been identified to carry even slight increases in the risk of developing cancer (e.g. genetic risk) is fueling the identification of exciting new chemopreventive agents. Some now argue that future development of chemopreventive agents offers greater potential for the long-term control of cancer than the much more widely studied and aggressively pursued chemotherapy agents. The basis for this optimism is seen in ongoing investigations that continue to reveal the step by step genetic and molecular basis of cancer development (especially early events). The emerging knowledge of the molecular mechanisms of cancer provides potential targets for specific agents that allow rational approaches to be devised for the chemoprevention of cancers.

The major long-term risk associated with radiation exposure during space travel is predicted to be radiation-induced cancer. The cancer-causing effects of low-LET radiations such as x-rays, g-rays, or electrons, typical of environmental earth exposures, have been relatively well-established. However, radiation likely to be encountered in space includes mainly heavy ions and protons along with their secondaries. Much less is known about the biology and risks associated with these types of radiation. The doses of radiation likely to be received even for long missions are probably low, but cover a broad range and are very unpredictable due to solar events. Like other types of radiation, the increased cancer risk associated with proton and heavy ion exposure is troubling because many radiation-induced cancers do not appear until later in life. Therefore, a large amount of uncertainty exists in how best to assess and manage the radiation risks associated with space travel.

Two high priorities in preparation for long missions are 1) providing a better understanding of both the short-term and long-term carcinogenic effects of heavy ion or proton radiation and 2) developing pharmaceutical countermeasures to mitigate the carcinogenic risk associated with low-dose and mid-dose exposures to these types of radiation.







As countermeasures to the cancer risk associated with space travel, chemoprevention offers a particularly promising avenue for investigation because of: 1) the difficulties associated with absolutely blocking radiation-induced mutagenic damage to DNA during prolonged space travel, either with shielding or pharmaceuticals, and 2) the prolonged latency period of most radiation-induced cancers (especially at low doses). This offers a prolonged time period when the most successful chemopreventatives exert their effects. For most cancers, compounds that modulate the regulation of cell growth and apoptosis (rather than blocking mutagenic damage to DNA) have to date shown particular promise in preventing overt cancer from developing in susceptible organs. Current chemoprevention successes against sporadic or familial tumors have identified tamoxifen for prevention of breast cancer, NSAID's (nonsteroidal antiinflamatory drugs) for prevention of colorectal cancer, and retinoids for preventing oropharangeal and other cancers as currently the top candidates for successful chemopreventation of specific tumors in humans.

Organs are not equally sensitive to the carcinogenic effects of radiation. Tissues that appear to be at higher risk for developing radiation-induced neoplasms include the female breast, the gastrointestinal tract (colorectal cancer), the thyroid, the bone marrow/lymphoid system (leukemia), and the lung. The female breast is particularly sensitive to the carcinogenic effects of radiation and therefore a relevant tissue in which to study chemoprevention of radiation-induced cancer. Furthermore, one of the most important advances in the therapy of breast cancer in the past two decades has been the development and use of tamoxifen for breast cancer chemotherapy. Over the past few years, tamoxifen has also emerged as an effective chemopreventative and now is the most widely prescribed anticancer drug in the world. It is prescribed mainly for breast cancer.

The class of compounds that includes tamoxifen, the selective estrogen receptor modulators (SERM's), are thought to have outstanding potential for use in estrogen replacement therapy and as chemopreventive agents. Burgeoning research and development of new SERM compounds has led to many new and improved SERM's undergoing trials. Tamoxifen, however, remains the prototype SERM for breast cancer chemoprevention. Newer SERM's will hopefully further improve on tamoxifen's effects while reducing its side effects. SERM's are ligands for the estrogen receptor (ER)

and modify carcinogenesis in breast epithelial cells by antagonizing ER signaling. However, in other tissues SERM's can act as partial ER agonists and promote the beneficial effects of estrogens in, for example, the skeletal and cardiovascular systems. Interestingly, tamoxifen may also affect carcinogenesis in a number of organ systems by disrupting apoptosis regulation in proliferating cells. In spite of the widespread use of tamoxifen, very little is known about its lifetime effectiveness against radiation-induced neoplasms-particularly those induced by radiation likely to be encountered in space such as protons and heavy ions.

Appropriate animal models provide a powerful means for directly evaluating the effectiveness of particularly promising chemopreventatives against cancers that may occur following radiation exposure. The rat mammary tumor model has been used extensively to analyze the carcinogenic effects of both chemical xenobiotics and physical agents. The Sprague Dawley rat mammary tumor model is particularly well-suited for studies in the low dose range because it is prone to develop mammary neoplasms early in life. Previous studies using the Sprague Dawley model have shown that sublethal doses of radiation (xrays, gamma rays, neutrons-not particularly relevant to space travel) induced mammary tumors, often within one year, and with a linear dose-effect relationship. Thus the Sprague Dawley rat mammary carcinogensis model not only closely resembles human breast cancer biologically, but it also is a highly sensitive model in which to examine the effects of radiation exposure and for testing pharmaceutical countermeasures against radiation effects. Our initial studies have focused on the effects of whole body, low level heavy ion and proton radiation along with chemoprevention of similarly induced mammary tumors using the female Sprague-Dawley rat mammary tumor model. Our rational approach to chemoprevention (SERM's) is based on one of the few successful emerging chemoprevention strategies used in human cancers to date in regard to sporadic or familial neoplasms. The well-studied, widely prescribed, protoypte SERM, tamoxifen has been effectively and safely used in humans for chemotherapy for almost two decades. These advantages, along with an understanding of its molecular mechanism of action suggests it would be an excellent candidate for successful long-term chemoprevention of specific proton and heavy ion-induced cancers. The prospect for successful long-term chemoprevention of this potentially important, late-appearing cancer relevant to space radiation exposure is indeed an exciting prospect.

Our hypothesis is:

If there is an increased risk for developing cancer due to radiation exposure during prolonged space travel, the increased cancer risk can be mitigated by chemopreventive countermeasures implemented during the long cancer latency period that follows radiation exposure. A logical and relevant area in which to test this hypothesis is in a radiation-induced breast cancer animal model since the female breast is one of the tissues most sensitive to the cancer-inducing effects of radiation and because recent evidence suggests that the compound tamoxifen is an effective breast cancer chemopreventative.

Our key findings thus far are:

Dr. Huso took over as PI of the chemoprevention studies less than two years ago and since that time considerable progress has been made in this area. Our studies are not complete, but preliminary evidence suggests that tamoxifen will be highly effective in preventing at least the mammary carcinomas that appear early following photon, proton, and heavy ion radiation exposure in the mammary gland. However, it appears there may be some variation in effectiveness depending on the dose and quality of radiation to which the individual is exposed. In addition, it is important to complete these studies and determine the effectiveness of tamoxifen for long-term chemoprevention of radiation-induced mammary cancer.

Although it is still early in the studies, preliminary results from our ongoing tamoxifen studies have pointed to a proof of principle for a strategy in which chemopreventive agents could play an important role in preventing breast cancer following exposure to radiation during space travel. This suggests that new chemopreventatives could be similarly identified that prevent other specific cancers associated with proton and heavy ion radiation exposure relevant to space exploration. Since cancer chemoprevention in general is still in its infancy as an emerging field, chemoprevention based on new targets and emerging compounds, hold considerable promise for continued improvement of strategies to effectively mitigate risks associated with radiation and other predisposing factors for cancers. Further studies are required to confirm the long-term safety and effectiveness of chemoprevention strategies, to identify additional agents that are effective against specific neoplasms, and to continue to improve chemoprevention effectiveness and implementation.

The implications of our findings for risk reduction for both space exploration as well as for the general population:

The implications are clear. Our results, though preliminary, provide a glimpse of the enormous potential payoff that chemoprevention research could provide in the battle against cancer. Regardless of the reason for an individual to be at increased risk for developing particular cancers, be it radiation exposure as in our studies (relevant to space travel) or genetic and enviornmental factors (relevant to the general population), specific chemopreventive compounds and strategies can be identified and implemented to mitigate risks that predispose individuals to cancer. Much work remains to be done to fully realize the benefits of chemoprevention strategies in the battle against cancer. Support for research into chemoprevention of radiation-induced neoplasms such as that provided by NSBRI therefore benefits not only space exploration efforts, but what is learned in this important area also could provide unique insight into cancer chemoprevention for the general population.

I.PROJECT RESEARCH ACTIVITY

A. Hypotheses, Objectives, and Specific Aims

Hypotheses

The hypothesis is that radiation damage leading to carcinogenesis can be mitigated by innovative countermeasures implemented following radiation exposure during the promotion and progression stages of cancer. A logical and relevant means to test this hypothesis is in the area of breast cancer since the breast is one of the most radiation sensitive organs in the body. Specific estrogen receptor modulators such as tamoxifen have recently been shown to be effective in the chemoprevention of breast cancer by inhibiting the promotion and progression of intitiated cells rather than acting prior to or during initiation steps.

Objectives

Overall the objectives were to establish risk estimates for protons and heavy ion radiations relative to low LET radiation and to determine if safe pharmaceutical countermeasures can significantly reduce the risk of developing breast cancer following such exposures.

Specific Aims

Specific Aim 1: To irradiate female Sprague Dawley rats with a range of doses of photons, protons, and iron ions appropriately chosen to be representative of the low level radiations relevant to space exposures. Following irradiation, the animals are being be monitored long term for radiation effects particularly focusing on tumor development in the mammary gland. The breast is particularly sensitive to the carcinogenic effects of radiation both in humans and in the Sprague Dawley model we have chosen. The doses which resulted in optimal tumor induction in aim 1 would then be used in future chemoprevention experiments in aim 2.

Specific Aim 2: To irradiate female Sprague Dawley rats with optimized doses of photons, protons, and iron ions that increase the risk of developing cancer in the mammary glands (aim 1). Control and tamoxifen-treated animals are being compared for tumor development in the mammary gland to determine if tamoxifen is effective as a countermeasure against radiation-induced breast cancer in the Sprague Dawley model.

In order to address the specific aims of the proposal several cohorts of the female Sprague Dawley rat mammary tumor model were irradiated at 60 days of age at Brookhaven National Laboratory (BNL) for heavy ions and Loma Linda University (LLU) for protons. As controls, a portion of each cohort was irradiated with photons at BNL, LLU, and JHU. A summary of the irradiated groups of animals is shown.

All groups are female Sprague Dawley rats exposed at 60 days of age

Radiation Effects Study

A) Initial Radiation Effects Study-currently > 2 years post irradiation

IRON ION EXPOSURES (number of rats)

200 cGy (18)
50 cGy (63)
16 cGy (71)
5 cGy (70)
Sham (70)

PROTON EXPOSURES (number of rats)

500 cGy (36)
160 cGy (36)
50 cGy (36)
Sham (36)

PHOTON EXPOSURES (number of rats) total irradiated at JHU, LLU, BNL

500 cGy (78)
160cGy (72)
50 (72)
Sham (72)

This group consisted of 730 animals. In ongoing studies, mammary biopsies and necropsy tissues have been collected, preserved, and are being analyzed. Mammary tumor biopsies are classified histopathologically as carcinomas or benign adenomas. Correlations are made between histopathology and radiation dose and quality.

B) Chemoprevention Study Controls

This group is of particular interest since they receive up to 25% dietary restriction to match their weight gains to the corresponding tamoxifen treatment groups. Dietary caloric restriction has been shown to significantly improve the longevity of rats. Therefore in this group a higher

percentage of animals should survive to old age and overall they likely will live longer.

Chemoprevention Controls for Radiation Effects Studies (no tamoxifen controls)

IRON ION EXPOSURES(number of rats/ current age in months) PROTON EXPOSURES(number of rats/age)

160 cGy (20/14 mo)	900 cGy (24/14 mo)
90 cGy (20/14 mo)	500 cGy (24/14 mo)
50 cGy (20/14 mo) (10/8 mo)	300 cGy (24/14 mo)
30 cGy (20/14 mo) (10/8 mo)	160 cGy (24/14 mo) (15/5 mo)
16 cGy (20/14 mo) (30/8 mo)	90 cGy (27/14 mo) (30/5 mo)
9 cGy (20/14 mo)	50 cGy (28/14 mo) (25/5 mo)
5 cGy (20/14 mo) (30/8 mo)	Sham (24/14 mo) 10/5 mo)
Sham (20/14 mo) (30/8 mo)	

PHOTON EXPOSURES (number of rats/age) total irradiated at JHU, LLU, BNL

500 cGy (30/14 mo) (20/8 mo)
300 cGy (30/14 mo (20/8 mo)
160cGy (30/14 mo) (20/8 mo) (15/ 5 mo)
90 cGy (30/14 mo) (30/ 5 mo)
50 cGy (30/14 mo) (20/8 mo) (25/ 5 mo)
30 cGy (30/14 mo)
Sham (30/14 mo) (20/ 8 mo) (10/ 5 mo)

The total number of rats that have been enrolled as irradiated chemoprevention controls (receive no tamoxifen) is 915. More than 90% of these are still being palpated and tumors biopsied. Lifetime analysis of these will be extremely useful for determining the radiation effects of

protons and iron ions, especially at low dose exposures.
All of the above animals were irradiated according to similar protocols. For the 14 mo old rats a single cohort of rats were shipped to each site and rats were irradited at all three sites (JHU, LLU, BNL) within a 10 day period. Animals were shipped back to JHU following irradiation and animals have been weighed regularly and subjected to 15-25 % dietary restriction to control the rate of weight gain. Additionally the rats are palpated when weighed and mammary tumors detected are sized and recorded. Tumors that grow are removed surgically as needed and all tumors are archived as frozen OCT blocks, formalin or methacarn fixed embedded tissues, or in RNA later for RNA preservation. Animals in good health are returned to the study after surgery. Rats are sacrificed as necessary according to health problems, but otherwise are kept for lifetime follow-up. Complete necropsies are performed and a histopathologic diagnosis is determined for all tumors. Analysis of other lesions is also underway.

Necropsy and Histopathology

Animals are identified by an eartag and by a subcutaneous transponder with a recorded, embedded unalterable random number crossreferenced with an assigned number with relevance to the animal's experimental group. All animals receive identification immediately following successful irradiation. Complete necropsies and microscopic examination have been performed on all at the time of death. At necropsy all organs and tissues have been examined for grossly visible lesions and examples of particular lesions photographed. All mammary tissue was carefully removed with the skin, examined, and then nodules removed for immediate analysis. The remaining mammary tissue was also preserved by fixation. All transponder chips remain with the appropriate archived tissues. Complete histopathologic examinations were performed on all mammary tumors. A database customized to our purposes is being developed (underway) with consultation from an on site expert. Individual animal records and results are accumulated in the database and easily retrieved for analysis of results. Our immediate goals are to fully analyze the information we are collecting regarding mammary tumors in the irradiated animals since that is the strength of the model system, and also obtain information about radiation effects in other organ systems in additional tissues we are archiving.

Chemoprevention studies

In 1998 the first large scale study demonstrating the effectiveness of tamoxifen chemoprevention for the prevention of breast cancer in humans was reported. Breast cancer has clear links to radiation exposure as the breast is one of the tissues most sensitive to the carcinogenic effects of ionizing radiation. Tamoxifen is not an ideal drug. It has been associated with uterine cancer and cataracts and was recently listed as a carcinogen itself. Yet it is the most widely prescribed anticancer drug in the world and it has demonstrated efficacy in preventing photon -induced tumors of the mammary gland and apparently has a wide margin of safety. Tamoxifen is the prototype of a family of estrogen receptor modulators that are the focus of intense developmental efforts. Therefore the chemoprevention studies examining the effectiveness of tamoxifen as a chemopreventative for iron ion or proton-induced mammary tumors may have far reaching implications as a countermeasure for the space program, especially as new, improved estrogen receptor modulator family members reach the market. Perhaps one of the most intriguing questions is, "Can tamoxifen prevent iron ion-induced or proton-induced mamary tumors that may appear much later in life after radiation exposure?"

A) Tamoxifen Chemoprevention Study: Analysis of experimental animals receiving tamoxifen chemoprevention following radiation exposure.

This group of rats receive continuous tamoxifen through an implanted slow-release pellet and they do not receive dietary restriction. Tamoxifen causes varying degrees of decreased weight gains so our goal in dietary restriction of controls is to match the tamoxifen-treated animals for rate of weight gain.

The following are the rats currently receiving conitnuous tamoxifen chemoprevention. Within each radiation dosage group, animals were weight-matched as pairs, and then split and assigned as either control or tamoxifen-treated animals.

IRON ION EXPOSURES (number of rats/ current age in months) PROTON EXPOSURES (number of rats/age

160 cGy (20/14 mo) 900 cGy (24/14 mo)

90 cGy (20/14 mo) 500 cGy (24/14 mo)

50 cGy (20/14 mo) (10/8 mo) 300 cGy (24/14 mo)
30 cGy (20/14 mo) (10/8 mo) 160 cGy (24/14 mo) (15/5 mo)
16 cGy (20/14 mo) (30/8 mo) 90 cGy (27/14 mo) (30/5 mo)
9 cGy (20/14 mo) 50 cGy (28/14 mo) (25/5 mo)
5 cGy (20/14 mo) (30/8 mo) Sham (24/14 mo) 10/5 mo)
Sham (20/14 mo) (30/8 mo)

PHOTON EXPOSURES (number of rats/age) total irradiated at JHU, LLU, BNL

500 cGy (30/14 mo) (20/8 mo)
300 cGy (30/14 mo (20/8 mo)
160cGy (30/14 mo) (20/8 mo) (15/ 5 mo)
90 cGy (30/14 mo) (30/ 5 mo)
50 cGy (30/14 mo) (20/8 mo) (25/ 5 mo)
30 cGy (30/14 mo)
Sham (30/14 mo) (20/ 8 mo) (10/ 5 mo)

The total number of rats that have been enrolled as irradiated animals receiving tamoxifen chemoprevention is approximately 915 also. More than 90% of these are still alive. This cohort is proving extremely valuable in determining the effectiveness of tamoxifen administration as a chemopreventative against the most relevant mammary tumors likely to be encountered following low-dose proton or iron ion exposure including those that occur later in life. A single cohort of rats was divided and were shipped to each irradiation site directly from a single supplier and rats were irradited at all three sites (JHU, LLU, BNL) within a 10 day period. For all the three different age cohorts, alls animals were shipped back to JHU following irradiation. One month after radiation exposure, rats were given tamoxifen at 200 ug/day (previously shown effective in preventing photon-induced mammary tumors) as a sustained release subcutaneous pellet. Unlike previous studies the animals then were switched to 20 ug/day for lifetime maintenance chemoprevention using 180 day sustained release, subcutaneous pellets. The rats have been weighed and

palpated regularly to detect mammary tumors. Tumors that grow to a designated size were removed surgically and all tumors archived as frozen OCT blocks, formalin or methacarn fixed embedded tissues, or in RNA later for RNA preservation. Animals in good health were returned to the study after surgery. Rats are sacrificed as necessary according to health problems, but otherwise are kept for lifetime follow-up. Complete necropsies are performed and a histopathologic diagnosis is determined for all tumors. It is important to get a clear idea of both the effects and effectiveness of long term tamoxifen administration. Tamoxifen resistant tumors will be compared with tumors of similar histological type from control animals and in some cases immunohistochemistry was used to assess estrogen receptor expression (see preliminary results). Additionally, immunohistochemistry for vimentin and cytokeratin were used to characterize certain tumors that were difficult to characterize by histological criteria alone. Although results are still not complete, one of the important questions is whether or not tamoxifen's effectiveness against radiation-induced tumors diminishes for tumors that appear later in life and how tamoxifen's effectiveness is influenced by the type of radiation to which the animals were exposed. These are important questions with direct relevance to reducing risks through countermeasures for the space program.

In summary

-Over 2500 total animals have been irradiated with protons, photons, or heavy ions relevant to space studies at the three different locations described and animals shipped back to JHU for enrollment in the mammary cancer chemoprevention studies. This large number increases the chances of finding significant, but subtle effects of low dose radiation and chemoprevention effectiveness at low radiation doses

-Over 700 complete necropsies on irraditate rats have revealed a variety of mammary and nonmammary, radiation-induced tumors

-Over 3000 total tumors have been surgically removed and are in the process of being analyzed of which over 1500 are carcinomas

-All relevant tumors and tissues are being archived for our ongoing studies on the effects and chemoprevention of heavy ion-induced and proton-

induced cancer.

Hence the data is truly unique in that:

1) it is directly relevant to space travel (heavy ions and protons),

2)the number of animals that are being studied (750 enrolled in year one, 1200 enrolled in year two, 600 enrolled in year 3) in order to more accurately examine effects of radiation at lower doses

3) the future plans to study multiple body systems (complete necropsies) for radiation effects from sexual maturity through aged animals (lifetime study) and

4) for the plans to test the hypothesis that chemopreventatives can be effective in mitigating risks associated with low dose radiation exposure for cancers that may occur later in life (effects of tamoxifen on breast cancer)

These studies are labor intensive because of the regular palpation of animals required, survival surgery procedures, complete necropsies and histopathology on all study animals, tissue preparation and archiving, and plans for administering tamoxifen continuously for the life of the animals.

Results-Radiation Effects

Histopathology

For our studies we have irradiated young virgin rats at approximately 2 months of age (sexual maturity). The developing mammmary gland in young virgin rats is composed of an interconnecting system of branching tubular structures lined by epithelium and ending as blind sacs terminally as structures called terminal end buds (TEB). The TEB's asynchronously differentiate over time and their development is sensitive to hormones of the female reproductive cycle. They progressively differentiate into alveolar buds and alveolar lobules. In the mammary gland, the number of proliferating cells is greatest and the cell cycle shortest in the least differentiated structure, the TEB, while the alveolar buds have the fewest proliferating cells and the longest cycle. It appears that the TEB plays an important role in giving rise to mammary carcinomas

while benign lesions such as adenomas, cysts, and fibroadenomas appear to arise from the more differentiated alveolar buds.

Histologically the mammary gland consists of compound tubuloalveolar glands which form irregular branching tubules with evaginations from their walls and from their blind ends. Secretory portions of the gland are located at the terminal portion of the branches. This terminal secretory portion is referred to as the ductule or alveolus. The ductules form a compact cluster around small intralobular ducts and are collectively referred to as the lobule, the functional unit of the mammary gland. These intralobular ducts join to form interlobular ducts which empty into the main lactiferous duct.

We have encountered non-neoplastic lesions in the mammary glands of the rats under study. These must be distinguished from benign and malignant neoplasms. Lobular hyperplasia consists of enlarged lobules of relatively normal appearing alveoli. The lack of a prominent collagenous stroma has been used in our study to differentiate these lesions from the fibroadenoma, a common radiation-induced benign tumor of the rat mammary gland. In atypical hyperplasia there is cellular atypia of the duct epithelium or alveoli along with papillary infoldings, arches, solid nests or plaques extending inward from the duct wall. Features of cellular atypia have included enlarged cells with vesicular or hyperchromatic nuclei. Cystic changes within the mammary parenchyma have been the most common non-neoplastic change which we have encountered. These thin-walled, epithelial-lined markedly dilated spaces often contain a granular, eosinophilic, secreted material or cholestrol crystals. On palpation, these cysts have been up to several millimeters in diameter and are soft and flucuent.

Fibroadenomas have been the most common benign neoplasm which we have encountered in the rat mammary gland. They are composed of abundant connective tissue, often densely packed, along with clusters of mammary epithelial cells. There has frequently been variation in the proportion of connective tissue to epithelial cells encountered in the rat mammary tumors examined to date. This has varied not only from tumor to tumor, but also on occasion within a single tumor. Fibroadenomas have had one of two patterns. A lobular pattern separated by dense layers of mature collagenous connective tissue. The ductules have been lined by a single layer of epithelium with small nuclei and a single nucleolus. A second pattern in some of the fibroadenomas has consisted mainly of

multiple concentric layers of densely packed connective tissue with a small number of widely dispersed ductules with attenuated or atrophic epithelium. On rare occasions we have had fibroadenomas which contained focal areas of atypia or even adenocarcinomas. Depending on the degree of atypia and growth pattern, these have been classified as carcinomas even though the majority of the tumor removed is a fibroadenoma.

Fibroadenomas have been a frequently encountered benign neoplasms in our irradiated animals. These, like carcinomas, are radiation-induced tumors even though they remain benign in their behavior. The benign fibroadenomas, however, continue to proliferate and grow and must be promptly surgically removed in order to follow irraditated female Sprague-Dawley rats for lifespan. The number and size of fibroadenomas, if not removed, eventually becomes a limiting factor for studies of aging, irradiated, female Sprague-Dawley rats.

Additional benign neoplasms which we have encountered are adenomas. Adenomas consisting mainly of glandular epithelial-lined acini with little stroma and with a tubular, secretory, or papillary pattern have also been removed surgically.

Radiation-Induced Breast Cancer

Mammary carcinomas (adenocarcinomas) are a radiation-induced malignant tumor that arises with increasing frequency during aging in our irradiated female Sprague-Dawley rats. These tumors have three main distinguishing features: 1) a loss of the normal tubuloalveolar pattern of the mammary gland, 2) cellular features of malignancy including cellular atypia, increased nuclear to cytoplasmic ratio, altered chromatin content, prominent nucleoli, increased numbers of cells in mitoses and abnormal mitotic figures, and cellular and nuclear pleomorphism. These tumors have frequently been accompanied by a prominent inflammatory infiltrate in the stroma. This usually has consisted of mainly mononuclear cells, but has also on occasion consisted mainly of eosinophils. In addition, a subset of these tumors are locally invasive through the tumor capsule or into surrounding muscle. Additionally, distant metastasis to the lung and other organs also occurs.

Radiation-induced adenocarcinomas have exhibited a broad range of patterns. The most common pattens have included:
1) Papillary pattern consisting of multiple branching papillae covered by one or more layers of cuboidal to columnar epithelial cells orented

perpendicular to the fibrovascular core.
2) Tubular pattern characterized by closely packed tubular structures which vary from round to elongated. The tubules have been lined by one or more layers of epithelial cells with the tubular lumina being small and empty.
3) Cribiform pattern has a sieve-like appearance in which sheets of epithelial cells have numerous secondary lumina or small round spaces filled with proteinaceous secretion.
4) Comedo pattern which is characterized by distended ductules filled with sheets of neoplastic epithelial cells and a central cavity filled with necrotic cells, cellular debris, and sometimes calcifying concretions.
5) Solid pattern is characterized by sheets of malignant cells that don't form well-defined acinar structures.

In older rats that have received irradiation, adenocarcinomas also arise within benign fibroadenomas. This is a rare occurrence in spontaneous mammary tumors, but appears to occur more frequently in the irrradiated animals. The tumors have mainly a papillary growth pattern of transformed epithelial cells.

Examples of the histopathology of many of these and additional tumor patterns of interest are included in the appendix.

Results-Chemoprevention

Pilot Tamoxifen Study

Dramatic recent research findings in other labs have focused new attention on a class of compounds known as selective estrogen receptor modulators. These agents behave as estrogens in some tissues, but block its action in other tissues. The prototype compound of this class, tamoxifen, has been shown in a large study, reported a little over two years ago, to prevent breast cancer in women who are at high risk for developing the disease. Prior to this no drug had ever been shown clearly to prevent the development of primary breast tumors. A related compound, raloxifene, used for osteoporosis treatment may also protect against breast cancer in a similar way. This is not only relevent to individuals with an increased risk of breast cancer due to increased radiation exposure during space travel, but it also is of importance to the general public. One of eight women will develop breast cancer during their life time. The breast is one of the most

radiation-sensitive organs in the body for the carcinogenic effects of radiation. Radiation exposure is a known risk factor for the development of breast cancer.

A pilot tamoxifen study for the prevention of radiation-induced mammary carcinomas was initiated using a limited number of animals (12) and photon irradiation. This was seen as an important step to take in preparation for large scale tamoxifen chemoprevention studies.

Twelve female Sprague-Dawley rats were irradiated with 500 cGy of photon irradiation. Following irradiation six of the rats received a timed release tamoxifen pellet containing 12 mg of tamoxifen to deliver 200 ug/day for 60 days. It was surgically placed under the skin. The animals were then palpated regularly to detect the development of mammary tumors. A summary of the results at 6 months following irradiation is shown. The results of this study showed that for early tumors, the six rats that received tamoxifen had only four tumors detected by palpation at sux months post irradiation. However, of the six rats that did not receive tamoxifen, there were 20 palpable tumors. These results suggested that tamoxifen was indeed an effective chemopreventative against radiation-induced breast cancer. Even though the rats received a known quantity of tamoxifen over a period of only 60 days, the tamoxifen had a significant effect in reducing the number of tumors that had occurred by six months following irradiation. Furthermore, tamoxifen administration was begun only after the rats had been irradiated since it acts during the promotion and progression stages of mammary cancer and is not known to prevent initiation or radiation-induced cellular injury. Our pilot study results support tamoxifen as a chemopreventative that could mitigate the increased risk of radiation-induced breast cancer that could occur due to increased exposure to radiation in space.

Large scale Tamoxifen studies:

As described previously large scale studies of the effectiveness of tamoxifen against mammary neoplasms induced by photons, protons, and iron ions are underway. The findings of the effectiveness of tamoxifen in chemoprevention of radiation-induced mammary cancer has been demonstrated also in preliminary results from large scale tamoxifen studies that are underway using the Sprague Dawley rat model.

Dietary Restriction

Recently it was shown that decreased weight gains that occur due to tamoxifen administration can have a significant impact on the rate of mammary tumorigenesis beyond the direct effects of tamoxifen. For this reason we placed all control animals on dietary restriction to match the average weight gains seen in the tamoxifen treated animals. The results of weight analysis is seen in a figure in the appendix.

Photon Irradiation

Continuous tamoxifen administration during the first year following photon irradiation was remarkably effective in preventing mammary carcinomas in this cohort. Without tamoxifen administration there was somewhat of a dose-response effect beginning to emerge where the carcinoma incidence correlated with the dose of photon irradiation to which the animals were exposed. These results are in the appendix.

Iron Irradiation

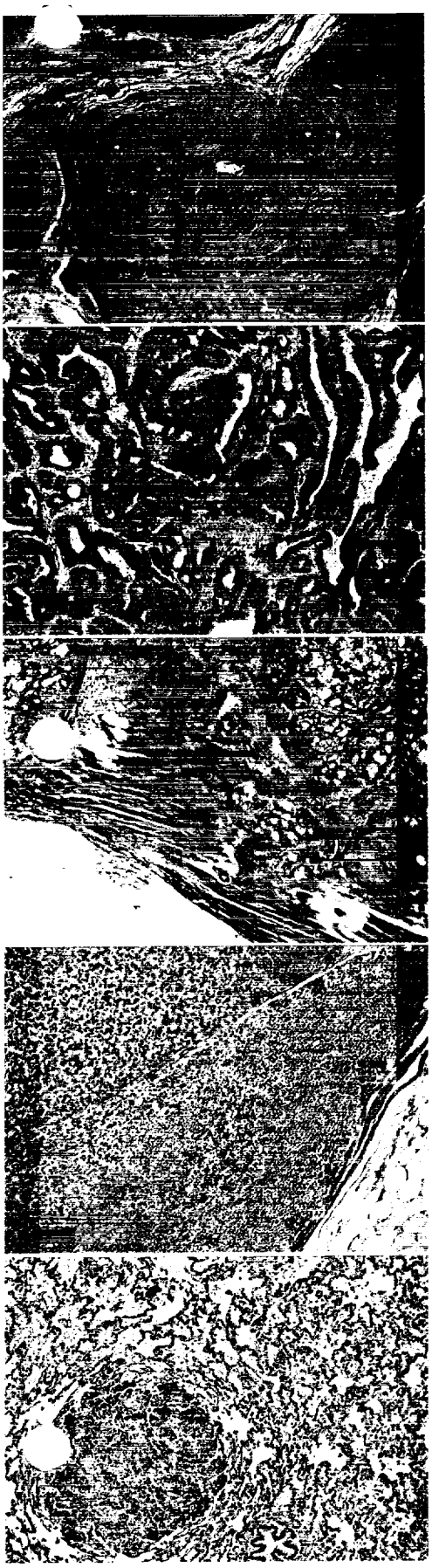
Continuous tamoxifen administration during the first year following heavy ion (iron ion) irradiation was very effective in preventing early carcinomas that arise during this period. A dose-response effect is emerging for irradiation dosage when correlated with the incidence of mammary carcinomas. The figure for these results is shown in the appendix.

Proton Irradiation

Interestingly, while tamoxifen was mostly effective in preventing proton-induced mammary carcinomas, tamoxifen was completely ineffective in preventing carcinomas induced in one of the high dose groups. It will be of interest to continue to follow these preliminary results over time to see if this trend continues. The reason for these findings are not clear at this point, since tamoxifen appears to be effective in reducing the incidence of early mammary carcinomas induced by low doses of proton irradiation.

Histopathology of Early-Response Mammary Tumors from Chemoprevention Study in Rats with Whole Body Exposure to Iron ions or Protons as Young Adults

(Huso et al, unpublished)



Benign Fibroadenoma

-Chemoprevention study; received 50cGy Iron ions as young adult..
-One month later, initiated continuous tamoxifen chemoprevention.
-Tumor removed 6 months after radiation exposure.

Fibroadenomas are the most common benign mammary tumor in our studies. Although their appearance is slightly delayed (months) compared to the appearance of mammary carcinomas following iron ion or proton irradiation, they eventually account for about 50% of the mammary tumors. They grow continuously, require surgical removal in long term studies, and are easily removed surgically.Note the stromal and epithelial components of the tumor.

Mammary Carcinoma, cribiform and papillary pattern

-Chemoprevention study; received 30 cGy Iron ions as a young adult.
-No tamoxifen (control animal); tumor removed 6 months after radiation exposure.

The papillary, cribiform pattern is the most common pattern of the mammary carcinomas. These are epithelial neoplasms with limited supportive stroma. The epithelial cells themselves often pile up, losing their normal orientation to the basement membrane and they often have enlarged nuclei with numerous mitotic figures.

Mammary Carcinoma, locally invasive

-Chemoprevention study; received 5 cGy Iron ions as a young adult.
-No tamoxifen (control animal); tumor biopsied 4 months after radiation exposure.

Histopathology of this carcinoma revealed that the tumor was invading the capsule and malignant epithelial cells extended into subjacent skeletal muscle. There has been no recurrence to date of this tumor or any tumor in this animal at 7 months post-surgery. The animal is being monitored closely.

Mammary Carcinoma, metastatic to regional lymph node

-Chemoprevention study; received 500 cGy Protons as a young adult.
-One month later, initiated continuous tamoxifen chemoprevention.
-Tumor removed at necropsy 10 months after radiation exposure.

At least during the early response, tamoxifen has been remarkably effective against radiation-induced tumors at most radiation doses tested. This is an example of a tamoxifen failure (not uncommon in human mammary tumors). This carcinoma grew in a solid pattern and here is shown following metastasis to a regional lymph node. We are evaluating its ER expression by immunohistochemistry.

Mammary Carcinoma, metastatic to lung

-Chemoprevention study; received 90 cGy Protons as a young adult.
-One month later initiated continuous tamoxifen chemoprevention.
-Histopathology of the lungs from necropsy at 9 months after radiation exposure.

This is another example of a tamoxifen failure during the early response period. Overall, tamoxifen has been remarkably effective as a chemopreventative even when initiated 30 days after exposure. However, tamoxifen-resistant tumors during the early response have been most common following proton exposures. ER status of this tumor is being determined.

II. IMPLICATIONS FOR FUTURE RESEARCH

The implications are clear. Our results, though preliminary, provide a glimpse of the enormous potential payoff that radiation-induced cancer chemoprevention research supported by NSBRI could provide both in support of extended space missions and in the battle against cancer. Regardless of the reason for an individual to be at increased risk for developing particular cancers, be it radiation exposure as in our studies (relevant to space travel) or genetic and enviornmental factors (relevant to the general population), specific chemopreventive compounds and strategies can be identified and implemented to mitigate risks that predispose individuals to cancer. Support for research into chemoprevention of radiation-induced neoplasms such as that provided by NSBRI therefore is of critical importance and benefits not only space exploration efforts, but what is learned in this important area also could provide unique insight into cancer chemoprevention for the general population.

Preliminary results from our ongoing tamoxifen studies have pointed to a proof of principle for a strategy in which chemopreventive agents could play an important role in preventing breast cancer following exposure to radiation during space travel. This suggests that new chemopreventatives could be similarly identified that prevent other specific cancers associated with proton and heavy ion radiation exposure relevant to space exploration. Since cancer chemoprevention in general is still in its infancy as an emerging field, chemoprevention based on new targets and emerging compounds, hold considerable promise for continued improvement of strategies to effectively mitigate risks associated with radiation and other predisposing factors for cancers. Further studies will be required to confirm the long-term safety and effectiveness of chemoprevention strategies, to identify additional specific agents that are effective against specific neoplasms, and to continue to improve chemoprevention effectiveness and implementation.

Other implications vary from scientific implications to programmatic implications for future research.

First our studies point out how animal models provide a powerful means to directly examine radiation effects and discover effective chemopreventation strategies. With the continued development of

genetically engineered rodents, it is likely that continued support of animal models as tools to use in risk assessment and pharmaceutical countermeasure studies will be crucial. However, their value is often overlooked due to their complexity and the expense associated with animal care. Adequate support for animal studies as well as support personnel for animal studies should continue to be a high priority. The data that animal studies provide is unique and provides critical information for risk assessment and effective countermeasure development that cannot be obtained in any other way when dealing with the effects of exposure to low levels of heavy ions and protons.

Second our studies highlight the strengths of supporting investigators with a combination of expertise. Radiation-induced cancer and chemoprevention studies in animal models are complex and requires a variety of expertise in order to carry out meaningful experiments successfully. Providing support for interaction between team members has enabled Dr. Dicello and Dr. Huso to work closely together toward a common goal. This type of interaction is difficult unless some type of mechanism is available to support interactive project studies as NSBRI has done in the past.

Third our studies point to the need for mechanisms to support more than 3 year projects. With our animal model studies, there is a lot of interest in the long-term effects of radiation during aging-often a long time after radiation exposure. While NSBRI to date has done an excellent job in continuing to support our long term studies, there is concern that certain important projects will be overlooked if they don't fit well into a 3 year project format. In addition, renewals always carry with them a degree of uncertainty and instability, especially for personnel (already trained) supported by a particular project. Availability of longer-term support would provide stability for support personnel which is a key issue in retaining persons with animal expertise and would benefit research outcomes of NSBRI supported research.

Finally, our findings provide clear evidence, though preliminary of the effectiveness of tamoxifen chemoprevention for preventing radiation-induced mammary tumors. Very few studies (perhaps only one) of chemoprevention of a radiation-induced mammary tumor model have been reported. Our studies have important implications for anyone receiving radiation treatment, accidental exposure to radiation, occupational exposure to radiation, or exposure during space missions. This

is especially true since tamoxifen is already widely prescribed in humans for chemotherapy.

In summary, there is very little known about the effects of these types of radiation, particularly the long-term effects of low doses of heavy ion and proton radiation. This information is critical in planning for space flight, especially long term space flight such as a trip to Mars. the implications for future research in our laboratories are that animal model studies evaluating the effects of protons, heavy ions, and photons are indeed feasible to perform on a scale large enough to achieve statistical significance. This is no small undertaking given that facilities that can reliably deliver desired doses of protons and heavy ions are at remote locations in relationship to our laboratories at JHU. The animals that have been irradiated, required sequential loading into individual animal holders, individual exposure to the appropriate beam and removal from the holders. The animals then need to be shipped back to JHU for lifetime monitoring of effects of radiation. These animals are extremely valuable and we have demonstrated an ability to keep the entire colony absolutely free of common intercurrent murine infectious diseases. This is an essential component in a lifetime study of this size.

CHEMOPREVENTION OF RADIATION-INDUCED RAT MAMMARY NEOPLASMS

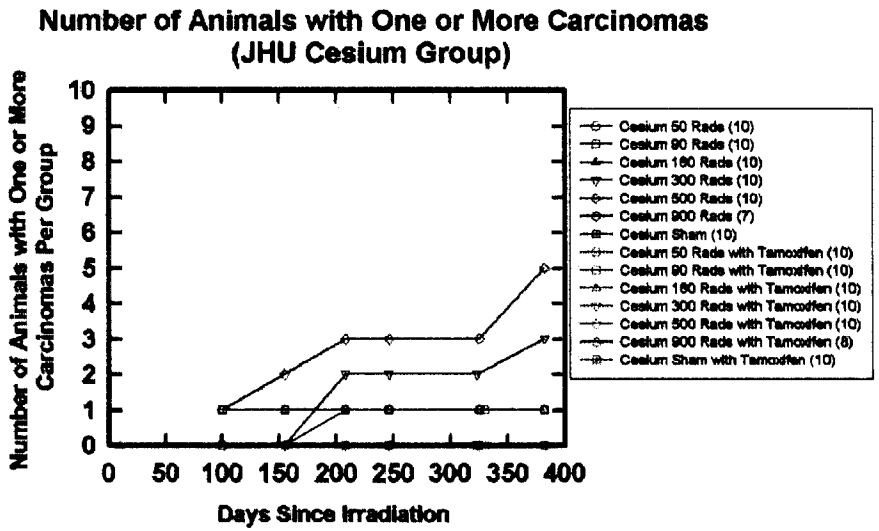
David L. Huso, PI

APPENDIX

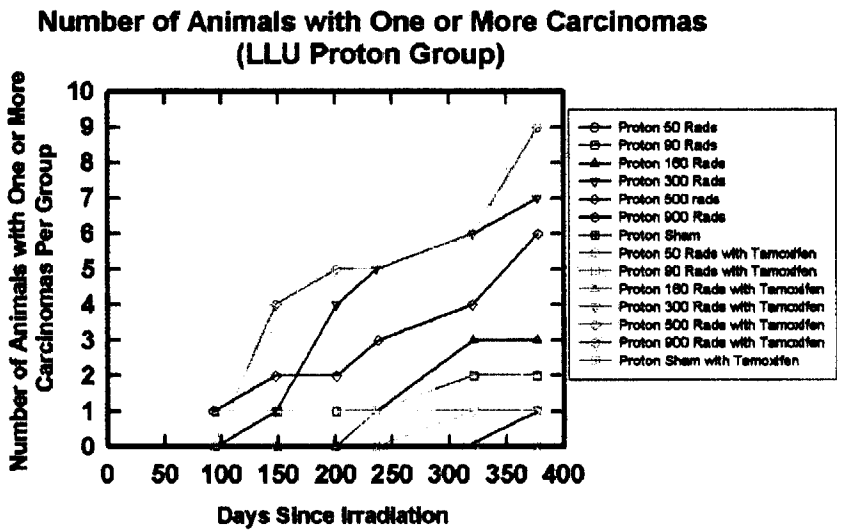
1) PROJECT RESEARCH DATA

2) ABSTRACTS AND PUBLICATIONS

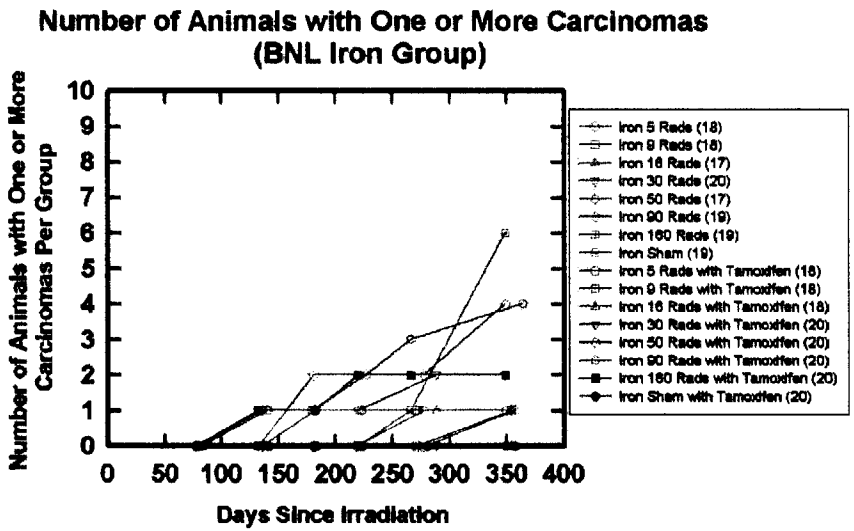
Chemoprevention Study: Tamoxifen effectiveness against the carcinomas that occur early following photon irradiation. Early carcinomas from this group were completely controlled by continuous tamoxifen admininistration. Long-term follow-up of this cohort will provide important new insights regarding continuous tamoxifen administration in the prevention of photon-induced mammary carcinomas. (Huso et al, unpublished)



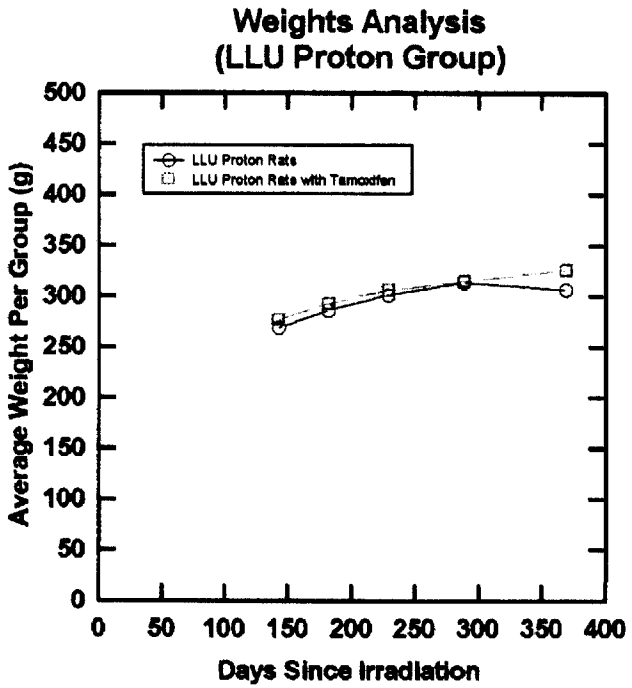
Chemoprevention Study: In this first group of animals irradiated with protons and treated with tamoxifen, tamoxifen was completely ineffective in preventing early respone tumors at one of the doses. The reason for this is still not clear at this point. An additional cohort of proton exposed animals is now also under study. The number of animals in each dosage group is normally shown in parentheses after each dose (It was inadvertantly left off here, but each dosage group represents carcinomas from 24-28 animals)



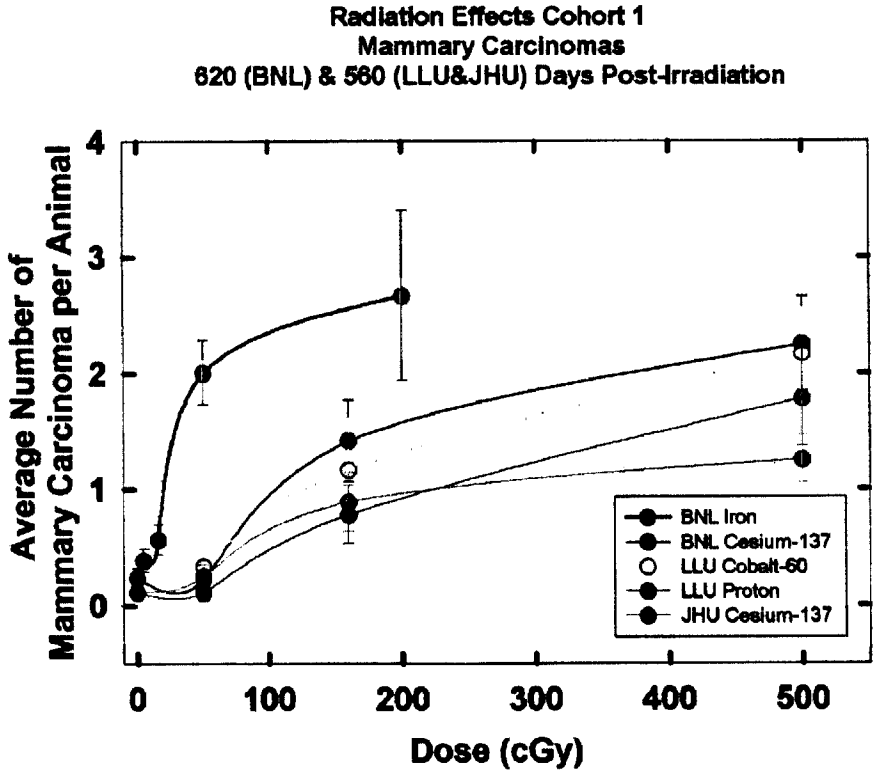
Chemoprevention Study: Tamoxifen effectiveness against the early response carcinomas that develop following iron ion radiation. This data is from the first cohort. An additional cohort for iron ion induction has been added to allow us to examine a larger total group of animals both during the early response period and later in life when low dose effects of irradiation are most likely to occur. These studies are ongoing.
(Huso et al, unpublished)



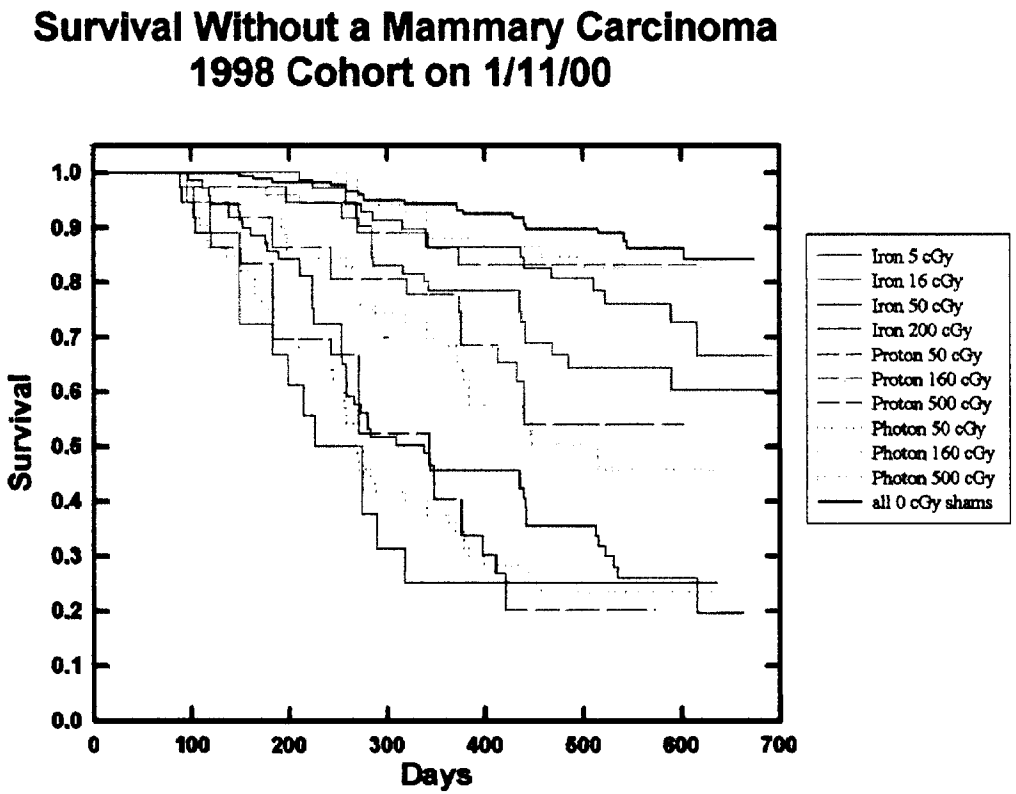
Chemoprevention Studies: Dietary restriction: All control animals in lifetime tamoxifen studies have been placed on dietary restriction and are monitored closely to maintain rats within 3-5% body weight range for each radiation dosage group. Since continuous tamoxifen administration reduces weight gain and differences in weight gain alone of ten percent can affect carcinoma development in rats, its important to keep control and tamoxifen-treated groups within this 3-5% range. That way the true effects of tamoxifen can be determined independent of effects of weight gain differences. This has only recently been demonstrated (from Grubbs CJ, et al 1999. Carcinogenesis 20:71-76) (data shown is from Huso et al, unpublished)



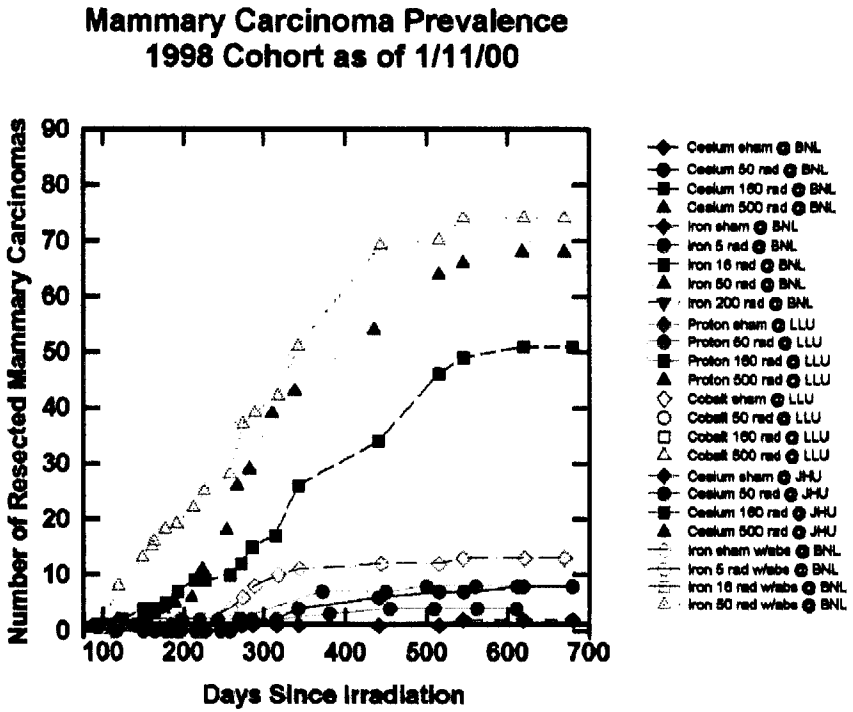
Radiation Effects Study: This is a summary of the first radiation effects study on the development of mammary carcinomas in a cohort of 750 female Sprague Dawley Rats that is now just over 2 years post-irradiation with photons, protons, and iron ions. Our experience in palpation, surgical biopsies, necropsies, animal husbandry, irradiation protocols at BNL and LLU, and shipping animals to JHU as well as database organization during the initiation and continuation of these studies has provided the basis to begin large scale studies to evaluate countermeasures against radiation-induced carcinomas and demonstrates the feasability of such studies.



Radiaiton Effects Study: This is the survival curve for the first group of irradiated rats. It shows that survival correlates with doseof radiation received. Our focus has been on mammary tumors, however, most animals do not survive beacause of other causes. We propose to analyze the nonmammary tissues in the proposed studies to determine the cause of death in the irradiated animals. Complete necropsies, reports, fixed tissues, and tissue blocks are being saved from all animals.

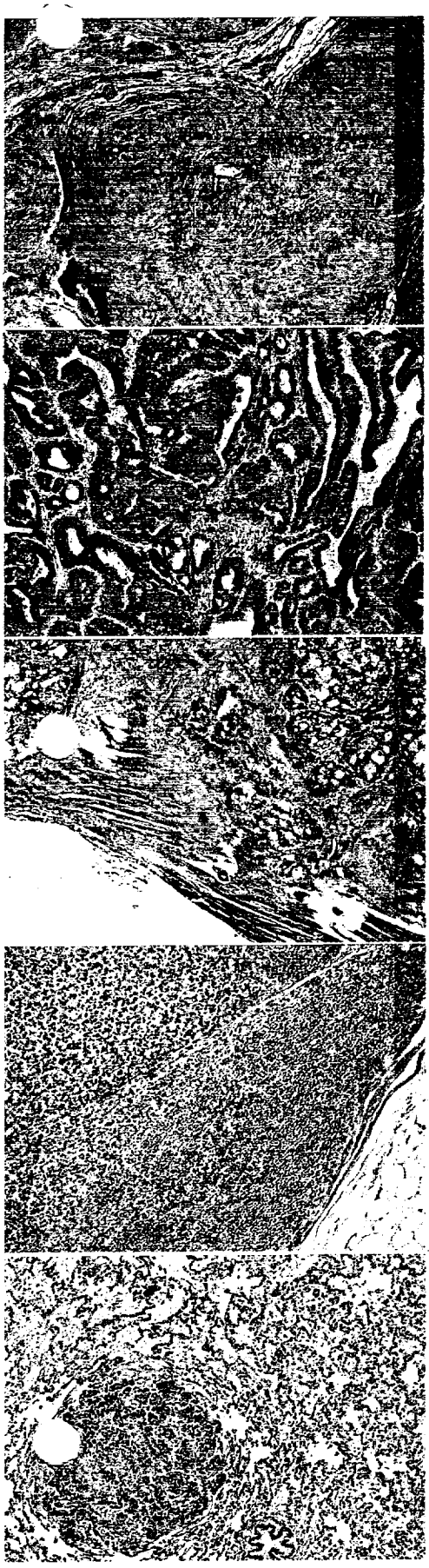


Radiation Effects study: This figure shows the dose response effect that is seen in the first cohort that was irradiated. These results show no tumor response at low-dose compared to background mammary tumor incidence. These results sugggested that chemoprevention studies of early response tumors may require mid-dose range exposure to protons and iron ions. Long-term follow up of low-dose and mid-dose groups would be required to determine what the late-occurring radiation effects would be in this model and if tamoxifen chemoprevention is an effective countermeasure against these late-occurring radiation effects.
(Dicello, Huso et al unpublished)



Histopathology of Early-Response Mammary Tumors from Chemoprevention Study in Rats with Whole Body Exposure to Iron ions or Protons as Young Adults

(Huso et al, unpublished)



Benign Fibroadenoma

-Chemoprevention study; received 50cGy Iron ions as young adult..
-One month later, initiated continuous tamoxifen chemoprevention.
-Tumor removed 6 months after radiation exposure.

Fibroadenomas are the most common benign mammary tumor in our studies. Although their appearance is slightly delayed (months) compared to the appearance of mammary carcinomas following iron ion or proton irradiation, they eventually account for about 50% of the mammary tumors. They grow continuously, require surgical removal in long term studies, and are easily removed surgically.Note the stromal and epithelial components of the tumor.

Mammary Carcinoma, cribiform and papillary pattern

-Chemoprevention study; received 30 cGy Iron ions as a young adult.
-No tamoxifen (control animal); tumor removed 6 months after radiation exposure.

The papillary, cribiform pattern is the most common pattern of the mammary carcinomas. These are epithelial neoplasms with limited supportive stroma. The epithelial cells themselves often pile up, losing their normal orientation to the basement membrane and they often have enlarged nuclei with numerous mitotic figures.

Mammary Carcinoma, locally invasive

-Chemoprevention study; received 5 cGy Iron ions as a young adult.
-No tamoxifen (control animal); tumor biopsied 4 months after radiation exposure.

Histopathology of this carcinoma revealed that the tumor was invading the capsule and malignant epithelial cells extended into subjacent skeletal muscle. There has been no recurrence to date of this tumor or any tumor in this animal at 7 months post-surgery. The animal is being monitored closely.

Mammary Carcinoma, metastatic to regional lymph node

-Chemoprevention study; received 500 cGy Protons as a young adult.
-One month later, initiated continuous tamoxifen chemoprevention.
-Tumor removed at necropsy 10 months after radiation exposure.

At least during the early response, tamoxifen has been remarkably effective against radiation-induced tumors at most radiation doses tested. This is an example of a tamoxifen failure (not uncommon in human mammary tumors). This carcinoma grew in a solid pattern and here is shown following metastasis to a regional lymph node. We are evaluating its ER expression by immunohistochemistry.

Mammary Carcinoma, metastatic to lung

-Chemoprevention study; received 90 cGy Protons as a young adult.
-One month later initiated continuous tamoxifen chemoprevention.
-Histopathology of the lungs from necropsy at 9 months after radiation exposure.

This is another example of a tamoxifen failure during the early response period. Overall, tamoxifen has been remarkably effective as a chemopreventative even when initiated 30 days after exposure. However, tamoxifen-resistant tumors during the early response have been most common following proton exposures. ER status of this tumor is being determined.

Johns Hopkins University

Histopathology of Mammary Tumors Arising in Older Rats with Whole Body Exposure to Iron ions and Protons as Young Adults (Huso et al unpublished)

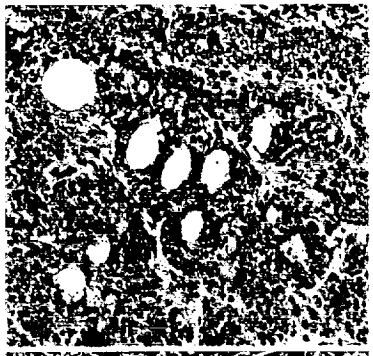
Radiation Effects Study. All tumors are classified according to the standardized system used by toxicologic pathologists. Studies of old animals may be especially important for examining low dose radiation effects and mitigation of these effects by chemoprevention.



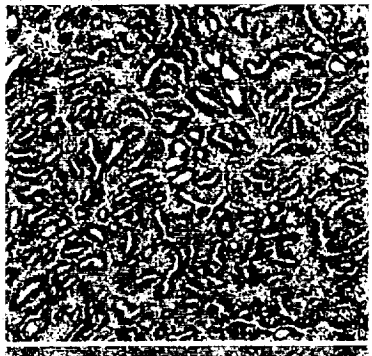
Benign cysts
Rat 25 months of age
Mammary gland
Whole body radiation
Protons:50 cGy as young adult



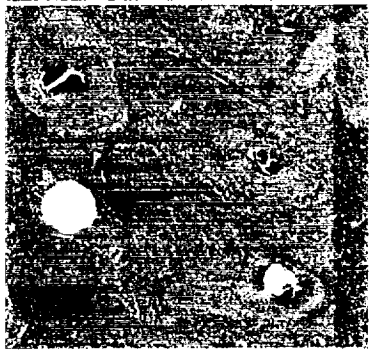
Mammary Carcinoma
Papillary and cystic
Rat 25 months of age
Whole body radiation
Iron ions: 50 cGy as a young adult



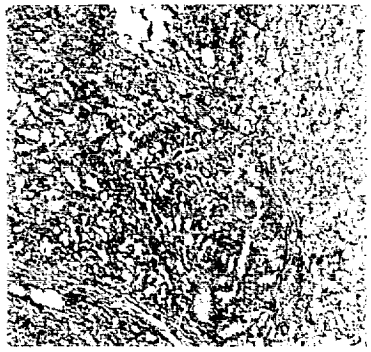
Mammary Carcinoma
Cribiform
Rat 25 months of age
Whole body radiation
Iron ions: 5 cGy as a young adult



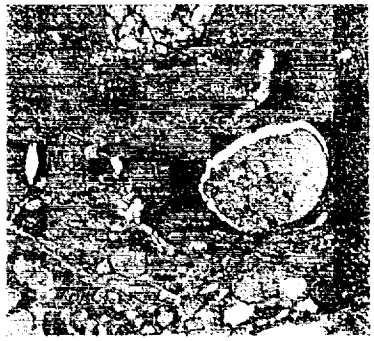
Mammary Carcinoma
Tubular
Rat 25 months of age
Whole body radiation
Iron ions: 5 cGy as a young adult



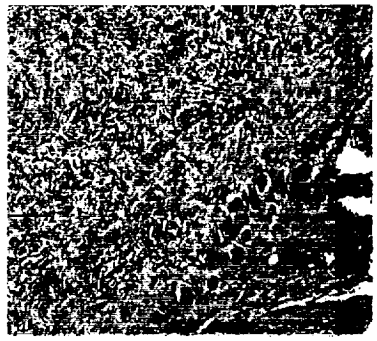
Mammary Carcinoma
Comedo
Rat 25 months of age
Whole body radiation
Protons: 50 cGy as a young adult



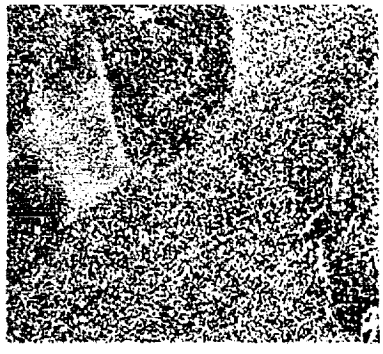
Benign Fibroadenoma
Rat 25 months of age
Mammary gland
Whole body radiation
Iron ions: 5cGy as a young adult



Mammary Carcinoma within a Fibroadenoma
Rat 20 months of age
Whole body radiation
Iron ions: 50cGy as a young adult



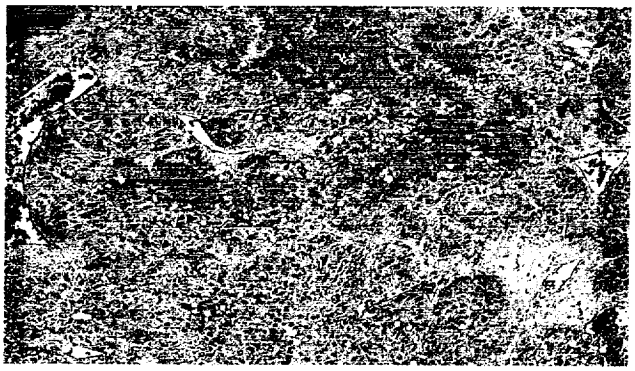
Mammary Carcinoma
Undifferentiated
Rat 17 months of age
Whole body radiation
Protons: 160 cGy as a young adult
Both epithelial and spindle cells appear neoplastic.



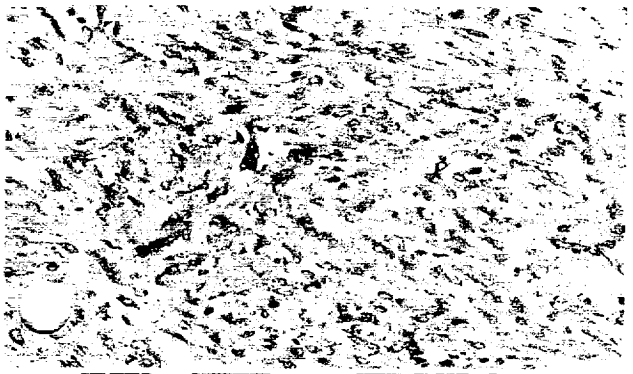
Mammary Carcinoma
Solid
Rat 17 months of age
Whole body radiation
Iron ions: 5 cGy as a young adult
Sheets of neoplastic epithelial cells are seen.

David L. Huso
Johns Hopkins University

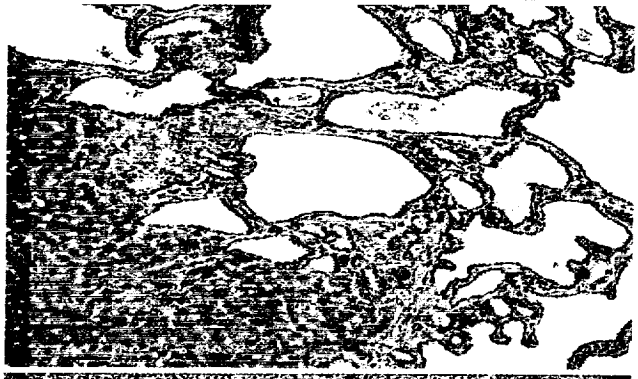
Non-mammary Tumors develop following Exposure to Iron ions and Photons



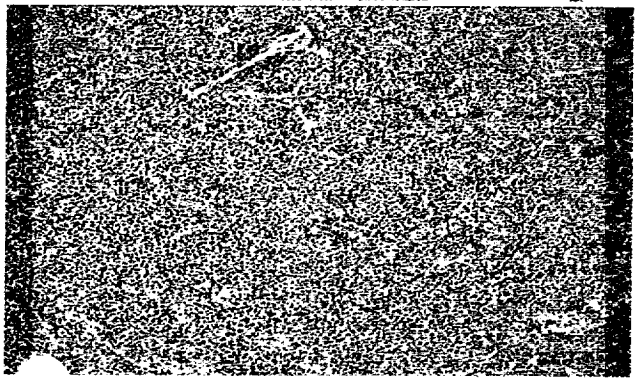
BRAIN-Pituitary tumor
Rat 21 months after exposure
Radiation Effects Study
Protons 50 cGy whole body irradiation



RIB-Osteosarcoma (fibroblastic)
Rat 12 months after exposure
Radiation Effects Study
Iron ions 50 cGy whole body irradiation



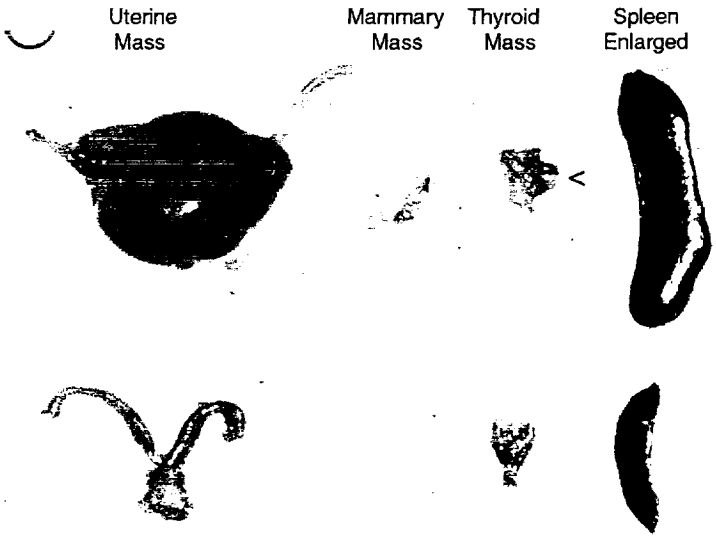
LIVER-Cholangiocarcinoma
Rat 23 months after exposure
Radiaiton Effects Study
Iron ions 200 cGy whole body irradiation



LYMPH NODE-Lymphoma
Rat 16 months after exposure
Radiation Effects Study
Iron ions 50 cGy whole body irradiation

Recent Examples of Radiation-induced Neoplasms in Old Rats Exposed to Protons and Iron Ions as Young Adults

(HUSO, ET AL UNPUBLISHED)

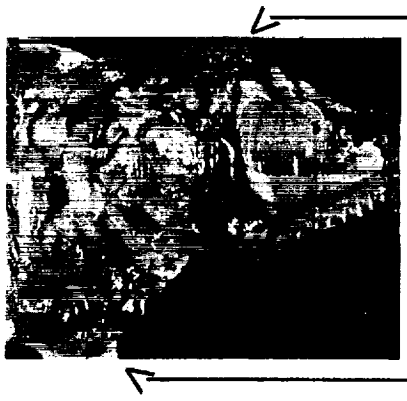


Chemoprevention Study
Female Sprague Dawley (SD) rat
12 months old
500 cGy Protons whole body radiation exposure as a young adult
Control rat (No Tamoxifen)

Normal Tissues



Radiation Effects Study
Female SD rat
24 months old
50 cGy Iron ions, whole body radiation exposure as a young adult



Chemoprevention Study
Female SD rat
14 months old
160 cGy Iron ions, whole body radiation exposure as a young adult
Control rat (No Tamoxifen)



Radiation Effects Study
Female SD rat
25 months old
200cGy Iron ions, whole body radiation exposure as a young adult

Manuscripts in Preparation

J Mann, J Dicello, D Huso, et al Comparison of continuous and short-term tamoxifen chemoprevention in a radiation-induced breast cancer model.

P Lombard, J Dicello, D Huso, et al Correlations between pituitary, radiation exposure and mammary carcinomas in a radiation-induced breast cancer model.

D Huso, J Mann, J Dicello et al. Effectiveness of tamoxifen against early-appearing mammary carcinomas induced by radiation exposure in a breast cancer model.

ABSTRACTS-

J Dicello, FA Cucinotta, DS Gridley, SP Howard, GR Novak, R Ricart-Arbona, JD Strandberg, ME Vazquez, JR Williams, Y Zhang, H Zhou, and DL Huso. NSBRI Radiation Effects: Carcinogenesis in Sprague-Dawley rats irradiated with iron ions, protons, or photons. *Annual Meeting NSBRI,* Winter, 1998.

J Dicello, FA Cucinotta, DS Gridley, SP Howard, GR Novak, R Ricart-Arbona, D Simonson, J Strandberg, M Vasquez, J Mann, and DL Huso. Carcinogenesis in Sprague Dawley rats irradiated with iron ions, photons, or protons. First Biennial Space Biomedical Investigators' Workshop. January, 1999.

J Dicello, FA Cucinotta, DS Gridley, SP Howard, GR Novak, R Ricart-Arbona, JD Strandberg, ME Vazquez, JR Williams, Y Zhang, H Zhou, and DL Huso. NSBRI Radiation-Effects Team and Carcinogenesis in Sprague Dawley Rats Irradiated with Iron Ions, Protons, or Photons. Eleventh International Congress of Radiation Research, Dublin, Ireland, July 18-23, 1999.

DL Huso. Chemoprevention of Rat Mammary Neoplasms. Animal Studies Workshop. Houston. (March 99)

DL Huso, FA Cucinotta, DS Gridley, SP Howard, GR Novak, R Ricart-Arbona, D Simonson, J Strandberg, M Vasquez, J Mann, and J Dicello. Chemoprevention of Rat Mammary Neoplasms. National Space Biomedical Research Institute Retreat (Jan 10-13, 00)

J Mann, R Ricart-Arbona, A Christian, D Simonson, K Nachman, J Dicello, and

DL Huso. Characterization of mammary tumors resistant to tamoxifen chemoprevention in a radiation-induced breast cancer model. Oncology Forum, Johns Hopkins University, (June,00)

D Simonson, R Ricart-Arbona, J Mann, and J Dicello, and DL Huso. Comparison of continuous and short-term tamoxifen chemoprevention in a radiation-induced breast cancer model. Oncology Forum, Johns Hopkins University, (June, 00)







COVER PAGE

Radiation-induced Cytogenetic Damage as a Predictor of Risk to Fe-ions and Protons

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CO-INVESTIGATORS: Constance Griffith, M.D.
Yongang Zhang, M.D.

EXECUTIVE SUMMARY:

This project has made several key findings that describe, in detail, chromosomal damage induced by three model space radiations: photons, energetic protons and energetic iron-ions (Fe-ions). The large number of data produced provide new insights into the relative potency of these radiations to induce different types of aberrations in multiple cell types: human lymphocytes, human mammary cells, rat mammary cells (in vivo and in vitro) and multiple forms of human colorectal tumor cells that have specific modulations of cancer-relevant genes. Further, we have investigated the induction of these radiations when used in fractionated or protracted time patterns in human lymphocytes, these data providing a paradigm for extrapolation of data from acute exposure to other exposure patterns. Further, the analysis of multiple aberrations per cell may be a "signature" for the dose and quality of radiation that induced these damages. We observe specific changes in induction of chromosome aberrations in cells that are deficient in expression of p53, p21 and 14-3-3-sigma, demonstrating that early changes in cells may render them more susceptible to further genetic damage induced by the three model space radiations. These data also provide new insights into the mechanisms of molecular biology by which cells process radiation damage. We have also measured clustering of multiple aberrations in individual cells so that Poisson analysis can be used to consider the relative influence of radiation quality on multiple events. Finally, we have suggested a new model for the dose response of cells to these radiations, focusing on induced cellular processing of radiation damage. Our model, that we term the subalpha- alpha- omega (SAO) model, provides a new
structure for mathematical paradigms for testing whether chromosome aberrations can be used as a surrogate marker in estimating cancer risk. Our data on chromosome aberrations, when combined with the outcome of parallel studies in carcinogenesis in the Sprague-Dawley Rat, will provide a direct comparison between the rate of induction of chromosome aberrations in mammary epithelial cells with the rate of induction of mammary cancer in the same animal model over the same dose-ranges.

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APPENDIXES:

A. PROJECT RESEARCH DATA

B. LIST OF PUBLICATIONS

C. COPIES OF PUBLISHED PAPERS (ONLY ORIGINAL COPY)

PROJECT RESEARCH ACTIVITY

Our research activity is described in eight categories:

1. We have compared aberrations induced in different cell types by photons, protons and Fe-ions and defined five separate categories of aberrations based on cellular susceptibility.

When data for aberrations induced by the three radiations are compared across several cell systems, it is clear that analysis should be segregated into five types of aberrations: i) gaps and breaks in chromatids {G+B}, ii) acentric fragments {AcF}, iii) dicentrics and rings, {D+R}, iv) complex aberrations {CCA}, and v) premature centromere separation {PCS}. For each of these five types of aberrations, at least one cell type shows unusual susceptibility to one type of aberration but not others, demonstrating that the genotype can "uncouple" cellular processes that lead from initial radiation damage to manifestation of aberrations. For induction of CCA for instance, increased susceptibility is limited to a single cell line (SW1222) in which expression of p53, p21 and 14-3-3-sigma, as measured by Western analysis is not observed in unirradiated or irradiated cells. Similarly, increased levels of premature centromere separation is also observed only in a single cell line, a double knockout of p53 in a previously wt p53 cell. Thus our analyses will focus on induction of three types of aberrations: G+B, AcF and D+R.

2. We have documented common dose-response patterns across all cell types.

While there is substantial variation between induced aberrations in different cell types and between Fe-ions and photons or protons, a common pattern can be observed for dose-response patterns for B+G, and a separate dose pattern for AcF and D+R. Background levels and relative numbers of induced lesions is cell type specific but our data shows a general pattern for all cells for all radiations with Fe-ions showing increased efficiency for AcF and for D+R.

2a. We document a "plateau" type dose-response pattern for induction of G+B in all cell types for all three radiations.

For G+B there is a significant background level in all cells and small doses (0.01 Gy) of photons and Fe-ions induce significant increases. These increases reach a plateau of response at approximately 0.05 Gy and remain relatively constant over extended dose ranges. Three genetically-manipulated human colorectal tumor cell lines have increased background levels of B+G compared to their parent lines. Two of these cell lines (80S4 and 19S184) are double knockouts of CDKN1A (p21) in parent lines HCT116 and DLD-1 respectively and the third line (14-3-3-sigma -/-) is a double knockout of 14-3-3-sigma in HCT116. Interestingly, the line 379.2, a double knockout of TP53 (p53) in HCT116 cells and SW1222, a cell line that does not express p53, p21 and 14-3-3-sigma, do not show elevated background levels of B+G.

2b. We have documented a "three component" dose-response pattern for AcF and D+R in all cell types for all three radiations.

For AcF, double chromatid fragments of equal length without a centromere, and for R+D there is a relatively similar general pattern, however differential quantitative susceptibility between cell lines and between radiations suggest to us that we should consider these two forms of aberrations differently. This common pattern is best described as a three component pattern, each response sequentially observed as dose is increased. In some 15 of our dose response

patterns, there is an indication of additional structure at very low doses (below 0.05 Gy) however further statistical analysis must be performed before such structure can be confirmed in this low-signaloregion. The three-component pattern is clearly and most easily discernable after Fe-ion irradiation and for cell types that are more susceptible to the induction of these aberrations. The dose response pattern can be divided into three components for Fe-ions, each limited to specific dose ranges, and to three components for both photons and protons which is observed at higher doses, at least for transition to the second and third components. However the shape is similar: an initial rise, a subsequent decrease in rate and then a transition to a higher rate. For both AcF and R+D there is an initial increase from extremely low levels in most unirradiated cells to a change in the rate of induction at approximately 0.25 Gy for Fe-ions and approximately 0.5 for photons. We will refer to this range of doses as the subalpha response. This reduced rate dominates until approximately 1.0 Gy for Fe-ions and between 1.0 and 2.5 Gy for photons and protons. We term this range of response the alpha response. As our standard challenge doses for both photons and protons were given at 1.0 and 2.5 Gy, the exact definition of the dose at which the rate of induction accelerates is difficult to estimate for these radiations. This final rate of rapid increase for AcF and for D+R we term the omega phase. The omega phase varies considerably between cell types and for cells with certain genetic deficiencies.

3. We have shown that the dose-response patterns for induction of chromosome aberrations may correlate with the induction of other cellular endpoints and with induction of cancer.

We have demonstrated that the three component (subalpha-alpha-omega) model that we observe for the induction of chromosome aberrations may also underlie the patterns observed for cellular mitotic death, for induction of mutation and in-vitro transformation and for cancer and have published these correlations (Williams et alia, 1998). This paper is attached in appendix C. Cell types vary in their susceptibility to the relative induction of aberrations over the three dose ranges. We propose that comparisons between cellular and molecular endpoints and between cellular and molecular events and the induction of cancer will be best performed using this model.

4. We have documented the response of peripheral human lymphocytes to graded doses of photons, protons and Fe-ions.

We have measured response of human peripheral lymphocytes derived from 17 "normal " donors when exposed to photons, protons and Fe-ions in quiescence and then assayed for aberrations in the first post-irradiation mitosis. We have measured the dose response for photons in 6 different donors, for protons in 4 different donors and for Fe-ions in four different donors. We have established background patterns and show that relative response to irradiation is not correlated with existing background patterns. These patterns show that the concept of RBE is not particularly useful in analysis. This data base will be important in the interpretation of chromosomal damage induced in lymphocytes of astronauts during space exploits.

5. We have documented the response of three types of mammary epithelial cells to graded doses of photons, protons and Fe-ions.

We have measured the response of three types of mammary epithelial cells: the human established line MCF-10A and primary mammary epithelial cells of the Sprague-Dawley rat irradiated either in vivo and then transplanted for assay or irradiated in vitro after explant and

assayed in vitro. These studies were done at the same time and at similar doses to animal carcinogenesis studies with the same animal. Our data show that cell irradiated in vivo are less susceptible to radiation-induced chromosomal damage but exhibit the same general pattern of response. Similar to 4 above, the concept of RBE is not effective as an analytical parameter.

6. We have documented the response of human cells with genotypes relevant to multistage carcinogenesis to graded doses of photons, protons and Fe-ions.

We have measured the response of eight different human colorectal cell lines selected for their relevance to multistage carcinogenesis. Cell lines varied in their status in expressing p53 (wildtype, dominant negative or null), p21 (competent or deficient) and 14-3-3-sigma (competent or deficient) and for one cell line (SW1222) that does not express p53, p21 or 14-3-3-sigma. Cells with dominant negative p53 are less susceptible to the induction of AcF and D+R than cells with wildtype p53, suggesting a mechanism for the increased prevalence of mut p53 in human tumors. Cells with aberrant G2-M arrest (SW1222 and 14-3-3-sigma -/-) are hypersusceptible to some forms of chromosomal aberrations but not others. These data now offer a model system for more detailed studies on the molecular biology of DNA damage processing that leads to the different types of chromosomal aberrations. These data demonstrate that the genotype of particular cell types modulate the expression of all five types of chromosome aberrations that we have studied.

7. We have documented the response of human peripheral lymphocytes to fractionated and protracted irradiation compared to single acute exposure.

We have exposed quiescent human lymphocytes to either radiation delivered in multiple small fractions (10 fractions of 0.25 Gy or 20 fractions of 0.125 Gy each) or continuously for 10 hours at 0.25 Gy/hr or for 20 hours at 0.125 Gy/hr. Our data show that there is no sparing effect for either fractionation or protraction for the induction of B+G. However, for both AcF and D+R there is a substantial sparing for protracted irradiation but only a small, but significant, sparing effect for fractionation.

8. We have documented the relative potency of photons, protons and to induce multiple aberrations in individual cells.

In all experiments we have evaluated each mitosis for the induction of all types of aberrations observable in solidly stained chromosomes. Thus we were able to measure the distribution of multiple aberrations in each cell as a function of dose and as a function of radiation type. Since the number and spatial distribution of ion clusters will vary for the three radiations (photons, protons and Fe-ions), we sought to compare the frequency distribution of multiple events as a function of dose for all three radiations. We observe that photons compared to protons and Fe-ions are most effective per unit dose for the induction of multiple aberrations (1, 2, 3, 4, 5....12 aberrations per cell). Fe-ions were significantly less effective in the induction of such multiple events than either photons or protons. These data will require further and extensive analysis for a better understanding of the spatial distribution of DNA "hits" for the three radiations. We suggest that the relative number of multiple aberrations in cells may be a "signature" for the type and dose of radiation to which the cells were exposed.

II. IMPLICATIONS OF PROJECT FINDINGS FOR FUTURE RESEARCH.

The implications of the findings listed above for future research are several and important.

1. **Our data offer immediate comparison with animal carcinogenesis data to determine whether chromosome aberrations are an appropriate biomarker for cancer risk.**

The data produced in this project was part of a team effort to measure effects of model space radiations in production of chromosome aberrations (this project) and in parallel experiments, in the induction of cancer. One of the major problems for assessing the carcinogenic effect of different types of space radiations on astronauts has been the absence of animal experiments of sufficient breadth and depth to establish a database for comparison to the human data base on radiation carcinogenesis. The subsequent analysis of the data from this project and the parallel animal studies will partly rectify this deficiency. While data from a particular animal model is limited, the breadth of the present experiments provides a basis for more confidence in interpretation of the results and their relevance in human risk assessment.

2. **Our data offer new insights for mathematical models needed to analyze patterns induced cancer in animals and humans.**

Human carcinogenesis data is limited both in terms of the types of radiation that have been documented to cause cancer in humans; in the dose-time patterns to which humans have been exposed; and in the number of humans within the study cohorts; this last factor limiting precision of measurement. Experiments in animal carcinogenesis are also limited in practical terms in the number of animals that can be exposed which in turn limits the number of radiations and time/dose regimens that can be examined. Additionally the problem of whether a specific animal model is an appropriate for human response must be considered. Mathematical models have been used to extrapolate from animals to humans, from higher doses to lower doses and from animal response to human response. Such models are based on postulating the shape of the dose-response curve so that higher doses that usually can be determined more precisely, can be extrapolated to lower doses that are more relevant to human exposure. Thus the mathematical model must postulate that response at higher doses is predictive at lower doses. The most widespread model, the linear quadratic model, postulates that only two parameters-the coefficient of a linear component and the coefficient of a quadratic component- are needed to predict the shape of the dose-response curve at all doses. Our data strongly suggests that this model does not describe what we observe as a more complex response pattern. Our data show that while there are indeed general response patterns, a three-component model, the subalpha-alpha-omega, model not only describes the dose-response curves for the induction of AcF and D+R in all cell systems and for all radiations, but also is consistent with patterns of induction of cellular events and cancer in animals. We are still in the process of developing this model in terms of the set of parameters that are sufficient to define the dose-response pattern over all relevant doses. Whether this model proves to represent better the underlying mechanisms or not, it is clear that it will be useful in comparing different types of radiations and different biological systems.

3. **Our data establish an important database for the response of different cell types to three model radiations.**

For instance, there is accumulating data on the prevalence of chromosome aberrations of different types in the peripheral lymphocytes of astronauts who have traveled in space. The data produced in this project will offer an excellent basis of comparison for these data. Similarly, the induction of chromosomal damage in other somatic target cells in human is extremely difficult to measure in situ or ex vivo. Thus the measurement of damage in an accessible cell such as the peripheral lymphocyte must be evaluated for response of other somatic cells that are known targets for radiation carcinogenesis, such as mammary or colorectal cells. The database that we have accumulated directly compares the response of human lymphocytes to other somatic cells and compares human and rodent cell response.

4. **Our data demonstrate the need for extensive fractionation/protraction experiments these data show that fractionation and protraction, at least over the doses/dose-rates used, do not predict response to the space environment composed of different patterns of time/dose/LET.**

The space environment is extremely complex in terms not only of multiple types of particulate radiation that occur there but also in the patterns of dose and time that characterize radiation exposure. The concept of dose-rate as it is currently used through identifying a dose-rate-modifying-factor (DRMF) may be limited in describing the mixture of high LET which will have an extremely high instantaneous dose-rate, but a low average dose-rate. We approached this problem with limited experiments that compared the induction of multiple small fractions (0.125 and 0.25 Gy) to protracted irradiation at 0.125 and 0.25 Gy/hr. We observe the later to be mores sparing for the induction of chromosomal damage (at least AcF and D+R) than the former. While there is probably some size of very small fractions that when delivered repeatedly may be equally sparing as protracted radiation, it is clearly smaller than 0.125 Gy. The determination of the equivalence or lack of equivalence of small fractions and continuous irradiation needs to be determined to model molecular/cellular response to space radiation environments.

5. **Our data predict that the relative susceptibility to chromosomal damage and probably then to subsequent advancement toward overt carcinogenesis, will vary in cells that due to prior genetic changes are at different stages in multi-stage, multi-pathway carcinogenesis.**

Multiple genetic changes are needed to convert a normal epithelial cell to malignancy. Since many events, perhaps as many as 5 to 15 are needed in some types of cancer, the probability that induction of multiple events in the same cell are independent is highly unlikely. Thus there is considerable thought that some pre-malignant changes predispose the changed cell to further changes. We have measured the response of human colorectal tumor cells to the induction of chromosomal damage by three model space radiation. These cells vary in their expression of at least three genes that are altered in tumor cells: TP53, CDNK1A and 14-3-3-sigma. We show that changes in these genes later the susceptibility to the rate of induction of different types of chromosomal damage. These data imply that space radiations may have differential effect on somatic cells that are already partially transformed. It seems reasonable to hypothesize that all astronauts have a substantial number of somatic cells that have undergone some premalignant changes. It therefore seems required to

consider the relative impact of premalignant changes on the susceptibility of somatic cells to transformation to the overt cancer state by space radiations.

6. **Our studies establish a structure for studies on the molecular basis of chromosomal damage.**

Our studies described in 5 above also provide a useful first step in studying the molecular basis of cellular processing of radiation-induced damage. Since we have described the association of hypersusceptibility of cells with specific genetic deficiencies to specific types of chromosomal aberrations, extension of these studies to other types of cells with other changes in genes relevant to cancer is needed.

National Space Biomedical Research Institute
FINAL PROJECT REPORT

COVER PAGE

PROJECT TITLE: **Quantitation of Radiation Induced Deletion and Recombination Events Associated with Repeated DNA Sequences**

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EXECUTIVE SUMMARY

Background and Significance of the Project:

Effects of Radiation on Biological Systems

Manned exploration of space exposes the explorers to a complex and novel radiation environment. The galactic cosmic ray and trapped belt radiation (predominantly proton) components of this environment are relatively constant, and the variations with the solar cycle are well understood and predictable. The level of radiation encountered in low earth orbits is determined by several factors, including altitude, inclination of orbit with respect to the equator, and spacecraft shielding. At higher altitudes, and on a Mars mission, the level of radiation exposure will increase significantly. A significant fraction of the dose may be delivered by solar particle events which vary dramatically in dose rate and incident particle spectrum. High-LET radiation is of particular concern. High-LET radiation, a component of galactic cosmic rays (GCR), is comprised of a variety of charged particles of various energies (10 MeV n^{-1} to 10 Gev n^{-1}), including about 87% photons, 12% helium ions, and heavy ions (including iron) (NCRP, 1989).

These high energy particles can cause significant damage to target cells. The different particle types and energies result in different patterns of energy deposition at the molecular and cellular level in a primary target cell. They can also cause significant damage to other, nearby cells as a result of secondary particles. Protons, for instance produce secondaries that include photons, neutrons, pions, heavy particles, as well as gamma rays. Heavy ions deposit energy in a "track" in which the magnitude of the damage varies as the particle loses energy. Heavy ions produce secondary delta rays, or electrons. The distribution of damage through tissue is described by a Bragg curve which will be characteristic for different energies. Needless to say there are differences in the RBE of protons and α particles, see for example (Jenner et al., 1992; Belli et al., 1992; Goodhead et al., 1992).

High-LET heavy ions are particularly damaging to cells as they do continual damage throughout their track. Differences in these energy deposition patterns can significantly influence the nature of DNA damage and the ability of cellular systems to repair such damage. It has been suspected that these differences also affect the spatial distribution of damage within the DNA of the interphase cell nucleus and produce corresponding differences in endpoints related to health effects. The interaction of a single high-LET particle with chromatin has been suggested to cause multiple double strand breaks within a relatively short distance. In part this is due to the organization of DNA into chromatin fibers in which distant regions of the DNA helix can be physically juxtaposed by the various levels of coiling of the DNA (Holley & Chatterjee, 1996). This prediction was confirmed by the detection of the generation of double strand DNA fragments of 100-2000 bp following exposure to high-LET ions (including iron) (Rydberg, 1996).

While it is very clear that ionizing radiation can cause cytogenetic damage and cancer, relatively little is yet known about the mutagenic or carcinogenic effects of high energy HZE particles in cells, for review see (Yang & Craise, 1997). High-LET radiation produces proportionally more double-strand than single strand breaks compared with low-LET radiation (Roots et al., 1985; Lett et al., 1987). Double-strand breaks are likely responsible for the cytogenetic damage visible as chromosomal aberrations, transformation, mutations, and delayed cell death (Kronenberg & Little, 1989; Kronenberg et al., 1995; Yang et al., 1997).

Nearly one-third of the human genome is composed of DNA repeats, which include simple mono-, di-, tri-, and tetranucleotide repeats; widely separated small and large repeats; and inverted repeats. Mutations associated with repetitive DNA are a source of many genetic diseases and cancer. Therefore, understanding how the various kinds of repeats contribute to the disease burden and understanding the impact of DNA damage on repeat-associated genomic instability is important for human health. Such repeated DNA sequences are likely to be very prone to mutation following exposure to high-Z high-energy (HZE) particles during space flight. Cells in the direct line of the HZE particle sustain a high dose of energy while cells surrounding the primary tract sustain a lower dose of energy from the energetic delta rays (electrons) produced by HZE particles. Therefore, the nature and pattern DNA damage to cells in tissue upon irradiation with HZE particles is particularly complex. It is important to understand the types of mutational changes induced by both the HZE particles as well as the delta rays.

Given the high frequency of occurrence of repeated DNA sequences it is highly likely breaks or base damage from radiation will occur within these sequences. Moreover certain processes of repair and recombination involve the generation of free 3' ends in DNA and extended single-strand regions that expose repeats to recombination or primer template misalignments. Therefore, the molecular events that are responsible for cytogenetic damage (chromosomal breaks and rearrangements) and other mutations (point mutations, frameshifts, small deletions and duplications) in many cases will involve primer template misalignments. (Note that we have shown that the molecular mechanism for a hotspot for several +1 frameshift mutations involves intermolecular strand switch events (primer template misalignments) that occur specifically in during leading strand replication at a region containing DNA repeats (Rosche et al., 1998).) It is also possible that a cell sustaining substantial damage from a heavy iron particle hit may saturate some or all of its repair capability, or induce an error prone mode of repair to mediate survival. The types of assays we are developing will provide sensitive reporters of the replication/repair fidelity of a cell following damage from HZE particles. It is the fidelity of this process which, if compromised, will ultimately lead to carcinogenesis or other detrimental effects of radiation damage. With the sensitive reporter constructs we are developing, the protective effects of chemopreventive measures or countermeasures can be quickly established. A major goal of this project was to provide a rapid way to test the efficacy of various countermeasures and chemopreventive drugs with respect to mutation minimization and cancer prevention. The sensitive, relative rapid assay being developed here would compliment the long term Dicello rat study being conducted at Johns Hopkins.

Goal:

The goal of this proposal was to develop data on the relationship between gene mutations, including deletions and recombination associated with direct repeats, and the quantity and quality of the radiation that interacts with the biological system so that countermeasures designed to minimize the health risks of radiation exposure in space can be devised. This goal could be accomplished by quantifying the rate of deletions between direct repeats, which may involve primer-template misalignment, recombination, or gene conversion in human cells following exposure to radiations which reproduce the energy deposition patterns produced in individual cells by the radiation environment in space. Using cell lines that provide sensitive reporters of mutations involving deletions between direct repeats and recombination events, we measured the rate of mutations in irradiated cells and in progeny of irradiated cells, following exposure to high energy alpha particles. We also planned to analyze several biological endpoints in other cells

lines that do not contain genetically selectable end points, but which contain long tracts of direct repeats (1.8 mb) or inverted repeats (15.3 kb). In addition, we also began developing additional reporter constructs for application in Sprague-Dawley ray mammary cells (and eventually rate) to increase the sensitivity of measuring deletions and recombination events mediated by DNA repeats. This will complement the long-term rat carcinogenesis study of the Radiation Effects Group, by providing a rapid, sensitive screen for the effects of chemopreventive and radioprotective drugs on genome instability following exposure to HZE particles and protons.

Hypothesis:

The hypothesis driving this proposal is that DNA damage introduced by high-energy (HZE) particles induces aberrant DNA repair events, involving repeated DNA sequences that lead to recombination, gene conversion, or other mutation, that initiate the sequence of cytogenetic and functional changes which manifest themselves as the long term health effects of radiation exposure in space, including cancer. Knowing the types of mutational events induced by different radiations will contribute to sound decisions for optimizing shielding and reducing biological consequences through use of radioprotective drugs or various countermeasures. The cell lines and procedures utilized in this proposal will be useful for testing the efficacy of various countermeasures and chemopreventive drugs.

Brief Summary of the Key Results to Date:

- The survival of four reporter cell lines (122-2, F14C-23, 7#7-7, and 3134) following exposure to 250 KeV X-rays has been measured. All cell lines were sensitive to X-rays in a range that would allow them to be used as reporter cell lines for radiation damage.
- The frequency of deletion of an inverted repeat and a nonpalindromic sequence from a *neo* gene in human 122-2 and F14C-23 cells have been measured (by isolating clones resistant to the antibiotic G-418) following exposure to 250 KeV X-rays.
- The nature of the reversion events, which involve precise deletions between direct repeats, has been analyzed by PCR analysis.
- The rate of reversion or the mutant frequency for the deletion mutations in the *neo* gene have been calculated for control and X-ray exposed cells. The frequencies are about $1\text{-}2 \times 10^{-7}$ in sham (non irradiated) cells. The rate increases by as much as a factor of 60 following exposure to X-rays.
- The survival and G-418 reversion frequencies following exposure to 1000 MeV Fe particles at the BNL4 and BNL5 runs have been measured. Following Fe exposure, the rate of G-418 reversion increases as much as a factor of 100.
- We have successfully cloned a 770 bp perfect inverted repeat (2 x 385 bp Alu sequence) and a 763 bp inverted repeat with a 39 bp nonpalindromic center (2 x 362 bp + 39 bp) in *E. coli*. This has taken considerable effort as this is 6-7 times longer than any inverted repeat we have previously cloned. A number of modifications to existing protocols had to be developed to get this. We are putting *Sph*I adaptors on it to clone it into the *neo* gene in pJJ999 (the vector be used for electroporation into rat cells.

Progress Toward Testing the Hypothesis and Fulfilling the Goals of the Project:

We have made good progress toward testing the hypothesis. However, this project lacked sufficient funds to make the kind of progress necessary to obtain its ultimate goals. Progress was slow due to the lack of sufficient personnel to devote full time effort toward all aims. Given the financial limitations, I feel we made excellent progress. We were able to confirm that the experiments we designed would work. This was evident from our results from the BNL4 and BNL5 radiations. Unfortunately, the pre-existing cell lines containing reporter constructs were not optimal for these experiments. Nevertheless, we were able to get the system to work and we correctly estimated the correct exposures for these experiments. Moreover, we learned the conditions necessary for the radiations.

Unfortunately, the project has come to an end just as we were at the verge of obtaining new reporter constructs (with the *alu* inverted repeats) that should work quite well for these experiments. More research and development was necessary in this area that was originally anticipated. The constraint of minimal personnel, slowed progress in this area.

Implications of the Results of this Project for Risk Reduction Related to the Critical Path and for Future Research on Earth Medical Problems:

This project was directed toward *understanding the molecular mechanisms of radiation damage and repair in cells.* These studies were designed to determine the relationship between the energy deposition pattern of radiation and its ability to increase the frequency of specific mutation events. DNA repeats are involved in many deletions and rearrangements associated with human disease. The reporter constructs, cell lines, and procedures to have been developed in this proposal will be directly applicable to studies on the effect of radioprotective drugs, as they would provide a very sensitive and relatively rapid quantitative assay for their effects. Moreover, these reporter constructs can be introduced into other types of cells and transgenic animals, including the Sprague Dawley rat. Thus, integration into the Dicello rat study may provide a way for ascertaining the effects of radiation and the protective effects of countermeasures, in a fraction of the time required for a long-term rat study. (Note, however, that the rat study must be completed for integration with a more rapid screen for countermeasures.)

Eventually our results will be useful for understanding the *genetic predisposition* to disease and cancer from radiation, which will be important for the potential genetic screening of astronauts.

Many other questions can be addressed with further application of our system, including those related to: chemical & biological agents that might be implemented to mitigate acute exposures, efficacy of radioprotectants, questions of shielding effectiveness, questions of fluence and fluence rate effects, and efficacy of nutritional supplements.

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I. PROJECT RESEARCH ACTIVITY

A. SUMMARY OF PROGRESS IN THE FIRST YEAR.

- In the first year we assembled and characterized four cell lines to be used in this study.
- We initiated measurement of the survival of four cell lines (122-2, F14C-23, 7#7-7, and 3134) following exposure to 250 KeV X-rays.
- The frequency of deletion of an inverted repeat and a nonpalindromic sequence from a *neo* gene in human 122-2 and F14C-23 cells was been measured (by resistance of clones to the antibiotic G-418) following exposure to 250 KeV X-rays.
- We developed PCR primers, DNA purification procedures, and protocols with which to analyze the nature of the G-418 reversion events by PCR and Southern hybridization.
- We began initial studies to look at the effect of alpha particles by measuring survival of 122-2 and 3134 cells following Cu-244 alpha particle exposure.
- We wrote a proposal to go to Brookhaven National Labs for the BNL4 run and treat our four cells lines with 1000 MeV Fe particles. This application was successful and Drs. Sinden, Ford and Braby went to BNL for training and a dry run. Then in May 1998, we exposed samples of all four cell lines to 0, 25, 50, and 100 cGy Fe.
- The survival and G-418 reversion frequencies following exposure to 1000 MeV Fe particles at the BNL4 run were initiated. It took this long to have the G-418 selection conditions optimized. We performed several analyses on 122-2 and F14C-23 cells.
- A 650 bp *Alu* inverted repeat was designed and Sprague-Dawley rat mammary cells lines were obtained for the development of new, more sensitive reporter constructs.

B. PROGRESS, RESULTS & ACCOMPLISHMENTS – YEAR 2-3

1. Four lines obtained for use in these studies.

Four different cell lines were irradiated during BNL-4 and BNL-5: F14C-23, 122-2, 3134, and 7#7-7.

1. F14C-23 is human fibroscarcoma cell line with a 122 bp inverted repeat (flanked by direct repeats) inserted into a *neo* reporter gene that is integrated into the chromosome (Kramer et al., 1996). Upon precise deletion of the inverted repeat the cells become resistant to the antibiotic G-418. The spontaneous deletion frequency is 1×10^{-8}, and the deletion frequency after 4 Gy X-rays is about 3×10^{-8}.
2. 122-2, is a human fibroscarcoma cell line with non palindromic insert flanked by direct repeats in the *neo* reporter gene (Kramer et al., 1996). The spontaneous deletion frequency is $<3 \times 10^{-9}$, while the deletion frequency after 4 Gy X-rays is as high as 8.3×10^{-6}.

3. Mouse lymphoblast cell line (7#7-7) contains a 15.3 kb inverted repeat (Akgun et al., 1997). This is a remarkable cell line, in that it contains the longest inverted repeat known to have been cloned. The inverted repeat is very unstable and it may be particularly unstable following exposure to iron particles. The frequency of genome instability is about 0.1 – 0.5 in progeny mice (Akgun et al., 1997). It also shows instability in culture (S. Lewis, personal communication). Genomic instability is detected by a change in the size of restriction fragments resulting from, typically an asymmetric deletion covering the center of the inverted repeat (Akgun et al., 1997).

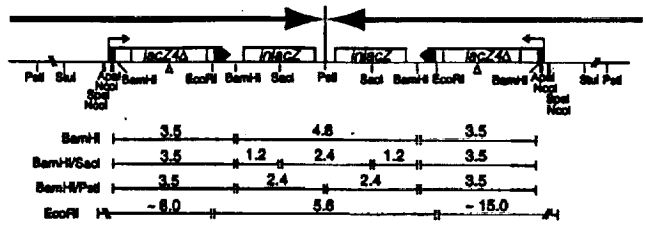


Figure 1: Structure of the 15.3 kb inverted repeat in cell line 7#7-7(Akgun et al., 1997). Genomic instability is easily detected by Southern hybridization using the 3.5 kb lacZ BamHI fragment (Akgun et al., 1997) and the enzymes shown in the bottom part of the Figure.

4. Mouse cell line 3134 is a mammary cancer cell line that contains 200 tandem direct repeats of a 9 kb hormone inducible gene (MMTV driven *ras* gene) (Richard-Foy & Hager, 1987). It was initially obtained for studies on the analysis of changes in DNA supercoiling and topological domain size in this region upon gene expression (Kramer et al., 1999). However, it provides an excellent model system to look at the genomic stability of a 1.8 mb region containing the 200 direct repeats following exposure to high-LET radiation. In collaboration with Dr. Hager's lab we planned to perform FISH analysis for genome rearrangement involving the 1.8 mb region. Metaphase plates hybridized with a *ras* probe reveal a large fluorescent region corresponding to the direct repeat region. The site of integration is near the end of chromosome 4, near a region of telomeric heterochromatin. We also planned analyze the structure of the repeats by pulse field gel analysis as we can examine the entire 1.8 mbp region as well as the structure of the collective 9 kbp repeats.

NOTE: Work on cell lines 7#7-7 and 3134 was not pursued after initial experiments in year #1 at the recommendation of NSBRI external review.

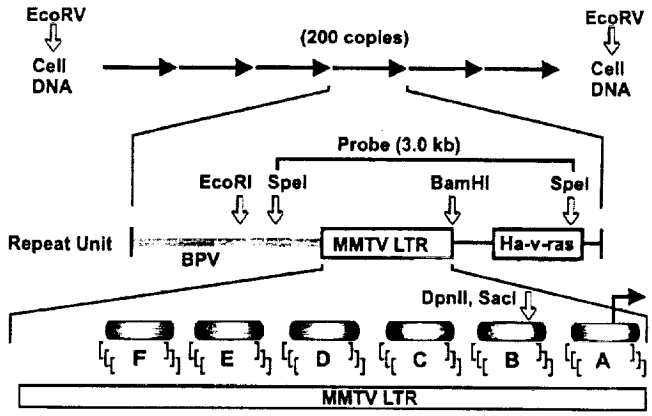


Figure 2: Genomic organization of MMTV-ras in the 3134 cell line. Plasmid pM18, containing both the MMTV LTR driving v-Ha-ras and the 69% transforming fragment of the bovine papilloma virus (BPV), was used to transform C127 mouse mammary cells to produce the 904.13 cell line with a replicating BPV-MMTV episome (Richard-Foy & Hager, 1987). The reporter sequence also includes viral like 30S sequences upstream and downstream of ras. The 3134 cell line is one of various lines derived from 904.13 where the episome has integrated into the genome. This cell line carries 200 copies of the integrated construct in a tandem array, as determined by primer extension [Fragoso & Hager unpublished observations], pulse field gel electrophoresis after restriction with EcoRV, which does not cut the array, and partial digestion with BamHI or EcoRI, which cut once within each element of the array. The sites recognized by SpeI in the BPV backbone and the ras gene, as well as the DpnII and SacI sites in the Nucleosome B region are indicated by arrows. The location of the hormone response element (filled rectangle) and the transcription initiation site (square arrow) are also illustrated.

2. Survival and Mutagenesis data for X-rays for the four cell lines.

We have measured survival to 250 kV/p X-rays for the four cell lines listed above. Figure 3 shows representative survival data for these cell lines exposed to 0-4 Gy X-rays. Cell lines 3134, 122-2, and F14C exhibit similar levels of sensitivity while cell line 7#7-7 was significantly more sensitive to X-rays.

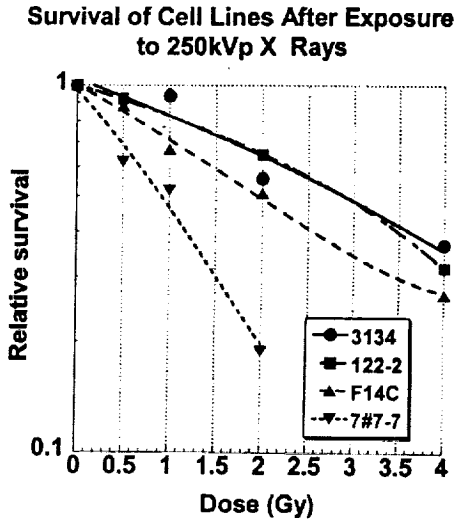


Figure 3: X-ray Survival Data

Following treatment with X-rays, selection with antibiotic G-418 was applied to cell lines F14C and 122-2 to measure the frequency of deletion of the inverted repeat and non-palindromic insert, respectively. Media containing 150 μg/ml of G-418 was changed every 48 hrs for >25 days to allow growth of G-418 resistant colonies. Figure 4 shows representative data for dose dependent accumulation of G-418 revertants following X-ray treatment. The number of revertants represents the total number detected on four 150 mm petri dishes. It is evident that there is a good dose response in that the number of G-418 revertants increased with increasing dose of radiation. From the survival data and the number of revertants, the mutation frequency was calculated.

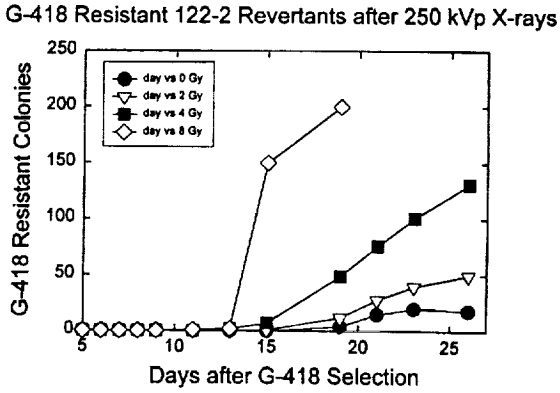


Figure 4 Accumulation of G-418 resistant revertants following exposure to X-rays.

Individual G-418 resistant revertant colonies were picked and cloned, and then the DNA was purified for PCR analysis of the region of the neomycin gene containing the 122 bp inverted repeat or non palindromic insert. Figure 5 shows representative results for a few G-418 resistant revertants from 122-2 following X-ray treatment. Two cell lines had complete deletions of the 122 bp mutation insert. One cell line, 122-2B2, 8 Gy, had a prominent full-length PCR product, indicative of the presence of the 122 bp mutation insert, although a fainter product corresponding to the deletion was observed. In this cell line an amplification of the *neo* gene region may have occurred with deletion of the 122 bp insert from one of the inserts. It is important to note that the original 122-2 cell line contained only a single integration of the *neo-hph* gene construct (Kramer et al., 1996). While the PCR analyses is in its initial stages, these results demonstrate that this is a viable approach and that it is working well for the G-418

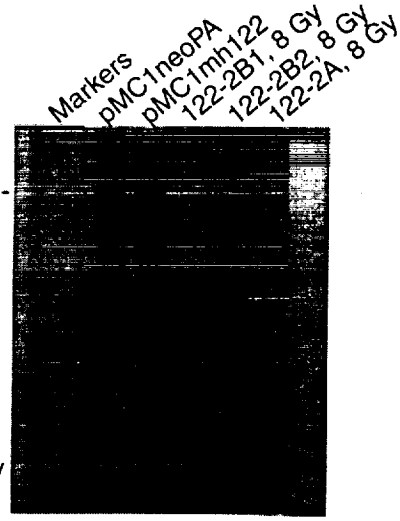


Figure 5 PCR analysis of 122-2 G-418 revertants following X-ray treatment. pMC1neoPA contains an intact neo gene. pMC1mh122 contains the 122 bp non palindromic insert in the NcoI site of the neo gene. The last three lanes show PCR products from G-418 resistant revertants arising following 8 Gy X-rays.

resistant revertant cell lines. This DNA has been purified using a protocol that works well in my lab for Southern analysis (Kramer et al., 1996), which will also be performed for G-418 resistant lines.

3. `Effect of Cu-244 alpha particle exposure on survival and deletion.

Cell lines 122-2 and 3134 were exposed to Cu-244 alpha particles at 1Gy/min, the survival measured, and cells selected for G-418 reversion. A comparison of the effects of Cu-244 and X-ray exposure is shown in Figure 6. As expected, Cu-244 particles were more effective in killing than X-rays for both cell lines.

4. Survival and G-418 reversion in cell lines 122-2 and F14C-23 following exposure to 1000 MeV/n Fe (results from BNL-4 & BNL-5).

After training for BNL-4 in 1998, Drs. Sinden, Braby and Ford went to Brookhaven National Labs and irradiated many samples of the 4 cell lines as reported in the 1998 Progress Report. Irradiation involved only frozen samples of F14C because the spontaneous mutation rate is so low that we may not have been able to detect many revertants irradiating samples on T25 dishes (applying selection to irradiated cells prior to growth and expansion of the culture). Cell lines 122-2, 3134, and 7#7-7 were irradiated both as frozen stocks and as living cells attached to T25 dishes or grown in suspension (7#7-7).

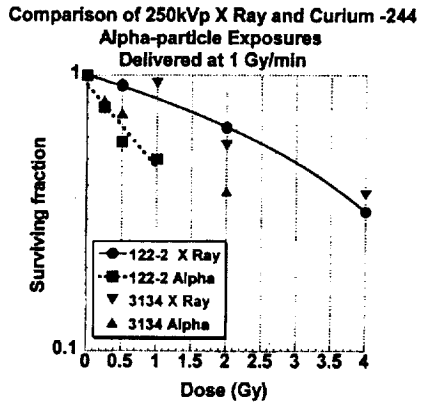


Figure 6: Survival following exposure to Cu-244 and Xx-rays.

On May 10-11, 1999 three cell lines (122-2 subclone A, 122–2 subclone B, and F14C-23) were treated with 0, 25, 50, and 100 cGy 1000 MeV Fe. These cells were treated attached to T25 flasks. The first set of plates (for all 3 cell lines) has been expanded and is under selection for G-418 reversion. Cells were also plated for survival analysis. The survival analysis is nearing completion. Cells from two other sets of plates were frozen for analysis at a later date. Also exposed to Fe were large samples of F14C-23 (thirty two 150 mm dishes) exposed in suspension. These cells survived nicely and have also been frozen for subsequent analysis. These experiments are critical as they represent a repeat of the experiment form a year ago. Moreover, during BNL5 all cells were exposed either on plates or in suspension. (No frozen cells containing DMSO were exposed.)

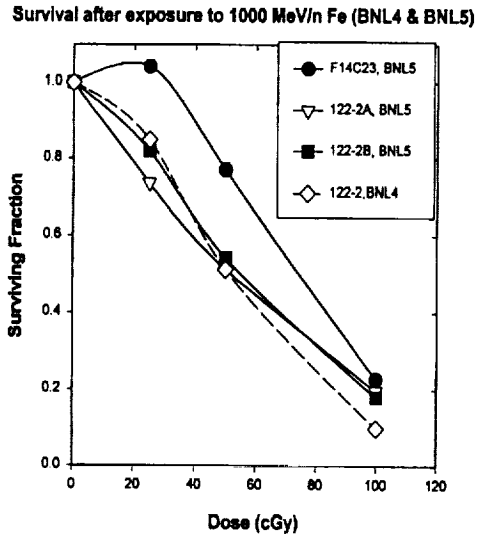


Figure 7: Survival following exposure to 1000 KeV/n Fe particles.

Figure 7 shows survival data for cells exposed to 1000 MeV/n during the BNL 4 & 5 runs. We were successful in measuring survival at doses equitoxic to those for the X-rays. G-418 selection data for one experiment for cells treated on plates during the BNL-4 run are shown in **Figure 8.** A reasonably good dose response was observed following heavy iron treatment. The results for cells irradiated as frozen cultures during the BNL-4 run were not encouraging; G-418 revertants were not strongly dose dependent and few revertants were detected. For this reason we decided

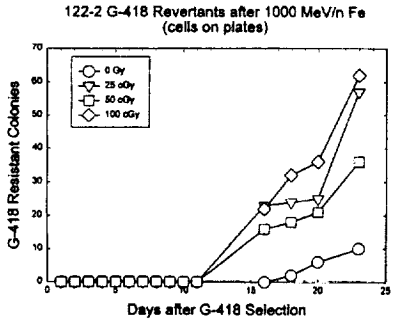


Figure 8: G-418 resistant revertants following 1000 MeV/n Fe. (BNL4)

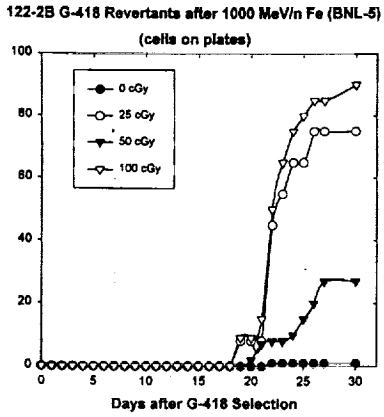


Figure 9: G-418 revertant cells; selection after first passage. (BNL5)

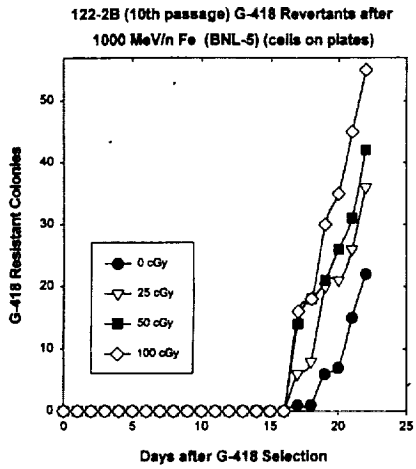


Figure 10: G-418 revertants after 10th passage of growth following exposure to 1000 MeV/n Fe.

to only treat cells on plates or in suspension for the repeat of this experiment during BNL-5. Results from BNL-5 G-418 selection are shown in **Figure 9**. Cells were also grown for 10 passages before selection was applied and these results are shown in **Figure 10**. These selections have been repeated several times. The results, to date, indicate that the instability associated with G-418 reversion in cell line 122-2, which involves deletion of the 122-2 bp non palindromic mutation insert occurs in a dose dependent fashion, although the response has shown some variability. The selection following growth for 10 passages simply indicates that the G-418 resistant cells persisted during unselected growth. In addition. we have isolated individual clonal cell lines that are still G-418 sensitive and then applied selection to determine if the cells, after recovery from DNA damage, continue to show a high level of genome instability. Revertants were rare in these analyses.

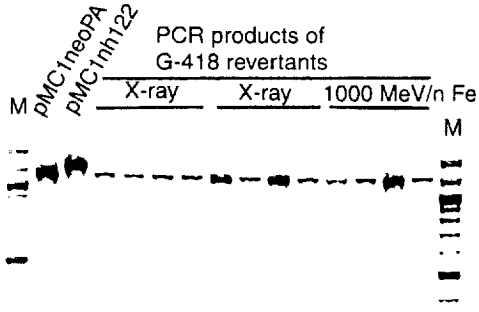


Figure 11: PCR analysis of deletion of the 122 bp insert in the *neo* gene.

To analyze the nature of the mutation leading to G-418 resistance, individual G-418 resistant revertant colonies were picked and cloned, and then the DNA was purified for PCR analysis of the region of the neomycin gene containing the 122 bp mutation insert. It is expected that G-418 resistance will result from precise deletion of the mutation insert, as observed previously for spontaneous reversion (Kramer et al., 1996). **Figure 11** shows representative results for a few G-418 resistant revertants from 122-2 cells following X-ray and Fe-particle exposure. The size of the PCR product is consistent with that for the original *neo* gene (lane pMC1neoPA), not that for a gene containing the 122 bp mutation insert (pMC1nh122). It is important to note that the original 122-2 cell line contains only a single integration of the *neo-hyg* gene construct (Kramer et al., 1996). Southern analysis of the G-418^r cell lines is planned to look for genome rearrangement, as observed previously (Kramer et al., 1996) (and to confirm the deletion) but these analyses have yet to be completed.

5. Construction and Purification of the Alu Inverted Repeat Constructs for Introduction into Human and Rat Cells

We have been successful in cloning a 758 bp perfect inverted repeat made of two 379 bp fragments containing the consensus human *Alu* sequence. In addition, we have cloned a 753 bp inverted repeat consisting of two 358 bp Alu-containing fragments flanking a 37 bp non

palindromic center. These sequences are six times longer than the longest inverted repeat we had made to date and it represents a considerable achievement as inverted repeats are notoriously unstable in *E. coli* (see for review (Sinden, 1994)). This was done by a two step cloning procedure introducing the *Sac*I-*Pst*I fragment containing the *Alu* consensus sequence from plasmid pPD39 (Batzer et al., 1994) into pUC19. The *Sac*I-*Eco*RI fragment was then introduced creating the perfect inverted repeat. For the quasipalindrome, the *Xba*I-*Pst*I fragment was first introduced into pUC19. As seen in **Figure 12**, plasmid AluP-7 contains a plasmid of the correct size as well as an insert consistent with 758 bp. A plasmid band corresponding to the parent pUC19 is also seen. This is consistent with deletion and, although this occurs, sufficient non deleted plasmid can be purified for the next cloning step. Plasmids containing the 753 bp quasipalindrome (with the 37 bp non palindromic center) are considerably more stable as seen in the lanes labeled AluQP-7 to AluQP-10 where the ratio of the insert-containing to parental (insert-deleted) plasmid is higher.

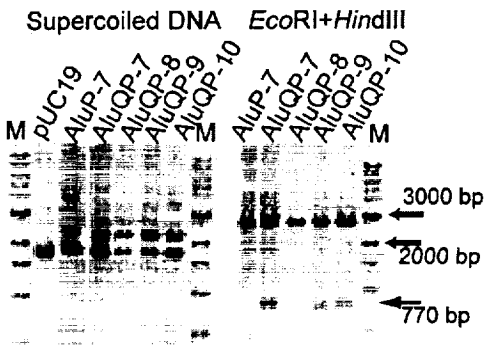


Figure 12: Analysis of Alu inverted repeats cloned into pUC19. Supercoiled DNAs (upper band in insert-containing plasmid while the lower band represents deletion products, uncut plasmids; *Eco*RI + *Hin*dIII, plasmid cut with those enzymes to generate the 770 bp inverted repeat. AluP-7, strain containing the perfect inverted repeat; AluQP-7-10, clones containing the inverted repeat with the 37 bp non palindromic center.

This is a very important result as it demonstrates that we can work with this inverted repeat in bacteria. This facilitates construction of the reporter cell lines. It is important to note, however, that the construction of the human cell lines could be done without cloning these plasmid in *E. coli.*

II. IMPLICATIONS OF PROJECT FUNDING FOR FUTURE RESEARCH

Implications for future research.

The types of assays we are developing will provide sensitive reporters of the replication/repair fidelity of a cell following damage from HZE particles. It is the fidelity of this process which, if compromised, will ultimately lead to carcinogenesis or other detrimental effects of radiation damage. With the sensitive reporter lines we are developing the protective effects of chemopreventive measures or countermeasures can be quickly established. Through the coordination of these studies in the Sprague-Dawley rat mammary cells with the studies for the Johns Hopkins rat study we intend to provide a rapid way to test the efficacy of various countermeasures and chemopreventive drugs with respect to mutation minimization and cancer prevention.

We hope to establish a model mutagenesis system in rat cells that reports genome instability as manifest by increased deletion and recombination events in rat cells following proton and iron particle radiation. In the future, the same reporter constructs used in the rat cells can be introduced into human cells and transgenic animals to provide further systems for establishing a cancer risk assessment for human space travel. Significantly, the information regarding risk assessment for space travel has important consequences for understanding cancer risk from radiation on earth.

An important point about our approach is that we are developing *sensitive genetic assays* for

instability, specifically deletions and recombination, that are likely primary events responsible for malignant transformation at the cellular level. These genetic assays allow measurements of instability (and thus cancer risk) to be made at relatively *low does*. This is important since exposures in space are at a low dose rate. By understanding mechanisms leading to instability, and being able to measure these at low dose rate, we will be in a better position to estimate cancer risk from exposure to radiation in space.

Eventually our results will be useful for understanding the *genetic predisposition* to disease and cancer from radiation, which will be important for the potential genetic screening of astronauts.

Many other questions can be addressed with further application of our system, including those related to: chemical & biological agents that might be implemented to mitigate acute exposures, efficacy of radioprotectants, questions of shielding effectiveness, questions of fluence and fluence rate effects, and efficacy of nutritional supplements.

Once our Sprague-Dawley rat reporter cell lines are constructed and characterized we can begin examining the effects of various countermeasures to reduce the risk of cancer and other deleterious effects of radiation exposure. To coordinate our efforts with the rest of the Radiation Effects Group we will begin a study of the efficacy of tamoxifen as a countermeasure. Tamoxifen acts not only as an antiestrogen, but it can be metabolized to compounds that form DNA adducts in rat (apparently less so in humans), it may act as a chemoprotectant, and it can influence p53 levels (Wei et al., 1998; Ye and Bodell, 1996(Guillot et al., 1996). Therefore, part of its chemopreventive effect may be other than its acting in an antiestrogen capacity. We have had initial discussions with Dr. Huso on strategies for tamoxifen experiments. We would coordinate these as closely as possible with the rat model system experiments.

Appendix:

A. Project Research Data: Lists of samples irradiated at BNL4 & BNL5, summary graphs of G-418 selections, etc follow this page.

B. Publications. None to date. Due to a very limited budget progress was steady but slow, and these experiments were 90% completed sufficient for publication at the time funding will expire for this work. Publication requires repeating results. These are long term experiments and access to the Fe beam is very limited to once per year. We hope to finished experiments this year to permit publication.

C. None

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NSBRI RESEARCH PROGRAM
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Feldmesser, H. S.	CO-I	Hopkins/APL	
Pisacane, V. L.	CO-I	Hopkins/APL	
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Sherman, D. A.	CO-I	MIT	
Potember, R. S.	**PI**	**Hopkins/APL**	**Miniature Time-of-Flight Mass Spectrometer**
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Maurer, R. H.	**PI**	**Hopkins/APL**	**In-Situ Spectrometry of Neutrons**
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NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

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FGY 2000 FINAL PROGRAM REPORT

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PROGRAM EXECUTIVE SUMMARY

The objective of the Technology Development Program of the National Space Biomedical Research Institute is to develop systems, instrumentation, devices, algorithms, etc., that are important to the work of the other Research Teams in the Institute and the at-large space life science community. The unique feature of the program's effort is the opportunity to bring an integrated engineering systems perspective to bear on technological developments to support basic research. Multi-disciplinary development teams have been established to work on strategically focused projects that integrate individuals with vastly different capabilities into a cohesive team.

Four development projects were selected, by independent review, for pursuit under the technology development program. All projects demonstrated excellent progress in achieving their individual goals and objectives. To preclude unexpected technology issues and assure that the projects would meet the needs of the other Research Teams, rigorous design reviews were conducted during the first year. Each project team was also encouraged to work closely with the specific Research Team what would benefit from their development. Designs have all been completed and prototype development accomplished with sufficient application to real problems to demonstrate utility. This portends ultimate success for the projects, though definitive science results are pending.

The Compact, High Precision, Multiple Projection DEXA Scanner project developed a concept for a low mass, volume, and power, high accuracy dual x-ray absorptiometer that will afford the ability to measure bone mineral density and geometry of the whole human body in space. This development supports the explicit needs of the Bone Demineralization/Calcium Metabolism Team. Acceleration of age related osteoporosis is rated as Risk Rank 1 of Risk Type I and the loss of skeletal muscle is rated at Risk Rank 1 of Risk Type II in NASA's Critical Path Roadmap. The project team has completed construction of a clinical engineering model and three-dimensional images of bone were successfully acquired. This device has greater precision and higher resolution than the latest commercial devices by a least a factor of three. It also has the ability to determine body composition of soft tissues important for the research of the Muscle Alterations and Atrophy Research Team, although this was a secondary objective. The value of this development is that a clinical engineering model has demonstrated the exceptional capabilities needed and that a flight instrument should have a mass of about 46 kg, tolerable by Space Station standards for a facility instrument. For the first time, the loss of bone mass, changes in bone geometry, and changes in body composition can be obtained throughout the whole body as a function of time during spaceflight. Whole body measurements are important because of the relatively poor correlation obtained between sites for age related osteoporosis and that bone loss in space may have different correlations. This will provide a better understanding of the basic processes at work, help assess the value of different countermeasures, and provide a method to mediate the application of the countermeasures eventually selected. System requirements for high-spatial resolution and rotational-scan geometry permits the system to provide the additional in-flight capabilities of digital radiography and x-ray computed tomography. The design is suitable for use in bed rest studies, the Space Station, and potential planetary missions. There has been extensive collaboration with the Bone Demineralization/Calcium

Metabolism Team and one of the Co-investigators of this project is an Investigator on that team.

The Instrumentation for Non-Invasive Assessment of Cardiovascular Regulation project developed instrumentation to non-invasively apply cardiovascular systems identification (CSI) to identify mechanisms responsible for cardiovascular regulation and alterations. This project directly addresses the needs of the Cardiovascular Alterations Research Team and may be used to support studies conducted under other team protocols. Impaired cardiac response is rated as Risk Rank 1 of Risk Type II in NASA's Critical Path Roadmap. The project team completed the development of an engineering prototype system that had been configured with data acquisition and processing applications. The final goal to automate all system operations has been accomplished and CSI utility has been demonstrated on human test subjects. The software developed in C++ uses the Windows NT 4.0 operating system on a Pentium II 300 MHz Thinkmate computer. This is a powerful approach to identify mechanisms responsible for orthostatic intolerance and to provide quantification of the baroreflex, autonomic function, and other physiologic control mechanisms under varying environmental conditions, such as microgravity. It can be used in space and has already had extensive application to human subjects on Earth. Other teams (Bone Demineralization/Calcium Metabolism Team, Neurovestibular Team and Human Performance Factors, Sleep and Chronobiology Team) may use the technique to measure changes in autonomic nervous function. In addition, this project has many applications to important problems in clinical medicine such the diagnosis and management of patients with diabetic autonomic neuropathy, heart failure, and hypertension. There is close collaboration as the Principal Investigator is team leader and a Principal Investigator in the Cardiovascular Alterations Research Team. The Cardiovascular Alterations Research Team anticipates using this instrument in the study of astronauts before and after spaceflight and ultimately, during spaceflight.

The Miniature Time-of-Flight Spectrometer project adapted a high resolution, portable time-of-flight mass spectrometer for quantitative measurement of human biomarker compounds in space flight. Applications include analysis of breadth, body fluids, products of infection, and perhaps DNA repair products and DNA mutations. As currently configured the system is of special value to the Bone Demineralization/Calcium Metabolism and the Muscle Alterations and Atrophy Teams but biomarkers important to several other Research Teams can also be obtained. Acceleration of age related osteoporosis is rated as Risk Rank 1 of Risk Type I and the loss of skeletal muscle is rated at Risk Rank 1 if Risk Type II in NASA's Critical Path Roadmap. The flight device will be inherently rugged, have mass of less that 5 kg, and will require less than 50 Watts. Identification of compounds with mass ranges of from 100 to 10,000 amu has been demonstrated. Key elements of this project were the development of a reliable sampling method and the quantitation of the measurements. The team has demonstrated the ability to routinely detect and accurately quantify (1-2%) 3-methylhistidine in urine, insuline-like growth factors (IGF-1), and estradiol. Membrane and electro-spray sample collection processes have been demonstrated. There has been close collaboration with the Bone Demineralization/Calcium Metabolism and the Muscle Alterations and Atrophy Teams.

The In-Situ Spectrometry of Neutrons project developed a portable, real-time neutron spectrometer, for the range of 10 KeV to 500 MeV, to support the needs of the Radiation Effects/DNA Damage and Repair Research Team. Carcinogenesis caused by radiation is rated as Risk Rank 1 of Risk Type I in NASA's Critical Path Roadmap. The

real-time neutron spectrometer prototype instrument is of portable brief case size, with a mass of less than 10 kg. Over the energy range of importance, it provides an energy resolution of 10 percent and also the counts for neutrons below 10 KeV. An alarm is included to warn astronauts when preset thresholds are exceeded. The project team has completed development of several test subsystems and an engineering model of the system, which includes two detector subsystems. The test subsystems have been submitted to characterization tests carried out with alpha particles at APL and with neutrons at different energies at Clemson University, Columbia University, and the National Institute of Science and Technology. The engineering model was reconfigured and has been evaluated on a high-altitude aircraft test flight at NASA Dryden funded by a small synergy grant. The engineering model performed as expected to an altitude where a short in the high voltage, due to a corona breakdown, terminated data collection. There has been close collaboration with the Radiation/DNA Effects Research Team and a Co-Investigator is a Principal Investigator in that team. Based on the accomplishments achieved in this project, the research team was awarded two NASA grants to use the instrument to characterize materials to minimize the production of secondary neutrons and to provide a neutron spectrometer for a flight to Mars to determine the safety of the Martian surface.

A Technology Development Working Group, composed of representatives from the other Research Teams, monitored and established technology development needs. The working group participated in teleconferences, team site reviews, and the integration of a technology requirements document (available under separate cover). This widely distributed document served to identify the needs of the Research Teams to a wide audience to help assure a broad and appropriate response to NSBRI calls for proposals to the specific needs of the NSBRI Research Teams.

The Technology Development Team embodies a sense of synergy that is unique to the Institute. There is a cohesiveness that exists between the individual project teams and researchers within other Research Teams. As well, there is a strong intra-team coalition that enables free and open technology interchange. All of these attributes provide a strong basis for contribution to, and support of, the Institute's mission. To assure synergy, there were 23 teleconferences, meetings with other Research Teams, and, in the last year, monthly lunches for the Baltimore/Washington Investigators.

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PROGRAM EXECUTIVE SUMMARY III

I. PROGRAM RESEARCH ACCOMPLISHMENTS 1

II. RISK REDUCTION ACHIEVED BY PROGRAM 11

I. PROGRAM RESEARCH ACCOMPLISHMENTS

The Technology Development Program of the National Space Biomedical Research Institute was established to develop products, including systems, instrumentation, devices, algorithms, etc., that are important to the work of the other Research Teams in the Institute and the at-large space life science research community. The activities also assist the transition of the respective technology to the civilian community to benefit society. The program's objectives are achieved through strong collaboration between the basic and applied researchers addressing the complex adaptation of humans to spaceflight and engineers, computer scientists, and physicists knowledgeable of state-of the art technologies. Multi-disciplinary teams have been established to work together on strategically focused projects that integrate individuals with vastly different capabilities into a cohesive team.

A Technology Development Working Group, composed of representatives from the other Research Teams was established to promote interest in the goals of the Technology Development Team and identify specific needs of the other Research Teams. This Group monitored and established technology development requirements, prepared the technology requirements document, and distributed this information widely. The working group participated in teleconferences, team site reviews, and the integration of the technology requirements document that is available under separate cover. This widely distributed document served to identify the needs of the Research Teams to a wide audience to help assure a broad and appropriate response to future NSBRI calls for proposals.

The program strategy of the Technology Development Team is to develop important systems and technologies to support the NASA Space Life Science Program with particular emphasis on working with and satisfying the needs of the other Research Teams in the Institute. It is also important that the existing technologies and capabilities of the entire research and industrial communities be fully exploited. The Technology Development Program has established and fostered cross-disciplinary, multi-institutional, multi-talented, strategically focused, and goal oriented project teams to advance technology developments to address the complex adaptation of humans to spaceflight. The specific objective of the Technology Development Program is to support the Institute Research Teams by developing systems, devices, instrumentation, algorithms, etc. necessary to:

(1) increase understanding of physiological and psychological responses to the space environment,
(2) develop countermeasures,
(3) support remote health maintenance and medical care,
(4) exploit the advances made to improve the quality of life on earth,
(5) support space life sciences educational and training programs, and
(6) promote technology transfer by collaborating with industry early in the development process.

An important NASA objective is that the Institute promote the transfer of its technology to civilian applications. By agreement, title to the technology resides with the institution in which the technology was developed. Consequently, the Principal Investigator has been encouraged to consider establishing partnerships with industry early in the development process so the licensing institution will have a potentially ready-made commercial partner. This would

reduce the time required to bring products to market and help ensure their availability to the broad research and civilian communities.

Project 1: Compact, High Precision, Multiple Projection DEXA Scanner
PI: H. K. Charles Jr., Ph.D.

This project used advanced sensor and detector design and fabrication techniques to develop a compact, storable, low mass, and low powered dual energy X-ray absorptiometer (DEXA) to determine at all body sites: bone mineral density, bone cross sectional area, and bone moments of inertia with a target accuracy of one percent and has the capability to measure regional composition of muscle and fat to about five percent. The effect of a microgravity environment on bone and muscle varies from one body site to another, so the ability to measure at all body sites was critical for success. This was especially so because the correlation between sites in age-related osteoporosis is not especially high and the correlation of bone loss in space may not be the same. The mass requirement for the system was less than 100 kg but the researchers had also set a non-commitment goal to reduce the mass to <60 kg. Through their research, a design with a mass of about 46 kg has been conceived. This instrument provides bone density and the structural properties of bones that are critical for understanding bone remodeling on the ground and in space. It also has the capability to measure the change in muscle mass necessary for understanding the process of muscle atrophy, but this was not part of the original proposal. This instrument can be used to investigate countermeasures for bone loss and muscle atrophy in space and then can be used to moderate the proposed countermeasure. System requirements for high spatial resolution and rotational scan geometry will permit the system to be extended to provide the additional capabilities of digital radiography and x-ray computed tomography. The design is suitable for use in bed rest studies, the Space Station, and potential planetary missions.

This project supports the explicit needs of the Bone Demineralization/Calcium Metabolism Research Team and the Muscle Alterations and Atrophy Team. The prototype system is capable of real-time monitoring of bone and potentially muscle loss at extremely high precision. Since the resultant measurements are patient specific, the system is useful for monitoring the effectiveness of countermeasures as well as determining the risk of fracture of individual astronauts under deployment scenarios. On Earth, the system is a natural adjunct to research on the effects of aging and disuse on bone integrity along with routine screening for osteoporosis and monitoring for efficacy of osteoporosis therapy.

Accomplishments:

- Engineering model hardware system has been successfully implemented.

A structured design strategy has been successfully followed to implement a fully functional clinical engineering model. The strategy has balanced the scientific challenges of high precision, whole-body, multiple-projection scanning with the engineering constraints of minimal volume and weight, reduced thermal characteristics, and low power utilization. Success has been achieved largely due to the systems engineering approach to the design and development. As such, state-of-the-art components and material selection have been employed throughout. High efficiency x-ray generation and detection techniques have been used to assure subject safety while reducing power and heat.

- Multiple-projection software for bone has been successfully developed and demonstrated.

Unlike commercial DXA systems, the clinical engineering model system is capable of both translation and rotation of the image plane. The objective of this implementation was originally to provide true three-dimensional bone geometry information, but, in addition, it affords the capability to directly determine magnification. To support the analysis of three-dimensional data, multiple-projection image analysis has been developed. Multiple-projection analysis enables the user to evaluate bone structural properties (e.g., bending strength) independent of subject position and orientation. Empirical evaluations to date have demonstrated an average coefficient of variation in the maximum and minimum moment of inertia (I_{max} and I_{min}) of 2.9% and 3.9%, respectively. It is projected that further processing refinement will reduce the error in a three-projection estimate to <1%; adding more projections will also reduce the error.

- High resolution and high precision monitoring capability has been demonstrated.

The engineering model system has demonstrated high spatial resolution imaging capability with a degree of quantization and signal to noise performance that far exceeds commercial system performance. Commercial DXA scanners employ pixels with dimensions of order 1 mm. The imaging panel of the engineering model has pixels with dimensions of 127 μm; geometric magnification results in an effective pixel dimension of about 82 μm at the plane of the subject. This performance level is a great advancement in meeting the requirements of the bone demineralization researchers. As well, the system has demonstrated the ability to distinguish soft tissue properties, thus affording potential use in muscle alteration studies.

- Bone reconstruction modeling techniques have been developed.

Multiple-projection imaging has led to the ability to generate three-dimensional reconstructions of the imaged bones. Future adaptation will permit full modeling with mechanical properties integrated with the shapes. Thus, this will allow risk assessment of astronauts to be done on an individual basis rather than on population means as now done with conventional DXA measurements which can sometimes be misleading.

- Weight reduction has been achieved.

Launch weight and spacecraft payload size limitations are serious factors associated with the viability of a piece of flight hardware. Without these constraints, commercial DXA systems are primarily designed for subject comfort and convenience. The project team has pressed hard to achieve exceptional system performance while also accommodating ease of use, minimum size, and a significant reduction in weight. The clinical engineering model was fabricated with existing components to meet the budgetary constraints, but a design exists of a system with a mass estimate of 86 kg, which is notably lower than the originally projected weight of 100 kg. In the 1-3 year time-frame, it is expected that advancements in x-ray tube technology will further reduce the weight to approximately 60 kg. In about 3-5 years, all of the component technologies (i.e., x-ray tube, detector, power supply, electronics) will have matured to the point where a target weight of approximately 46 kg will be achievable.

- Industry collaboration has been successful with the x-ray tube and detector.

Significant industry interaction and collaboration has been realized in this project. The project team has established a very close relationship with Varian Medical Systems for the development and evaluation of the x-ray tube and the detector array. The project team has had access to the latest technology developments and to pre-production hardware. This synergistic interaction has

contributed in great measure to the system's high resolution and high precision performance as well as the reduced system weight.

- An IRB has been approved for human studies.

In collaboration with the bone demineralization team, a human study has been proposed in which the clinical engineering model will be used to monitor changes in bone characteristics in mobility-limited subjects. IRB approval has been granted for the use of the system without any conditions imposed. The FDA has granted an Investigational Device Exception for use of the clinical engineering model for human studies.

Project 2: Instrumentation for Non-Invasive Assessment of Cardiovascular Regulation
PI: R. J. Cohen, M.D., Ph.D.

Instrumentation developed in this project is used to assess non-invasively cardiovascular regulation and alterations by means of the Cardiovascular System Identification (CSI) technique. CSI is a non-invasive means of quantitatively assessing closed-loop cardiovascular regulation by means of the mathematical analysis of second-to-second fluctuations in non-invasively recorded cardiovascular signals such as heart rate, arterial blood pressure, respiratory activity, and cardiac output. From this analysis, one creates a quantitative model of closed loop cardiovascular regulation for each individual studied. This is a powerful approach to identify mechanisms responsible for orthostatic intolerance and to provide quantification of the baroreflex, autonomic function, and other physiologic control mechanisms. Until now, CSI analysis has been cumbersome to implement and could only be conducted by expert users performing time intensive analyses of previously recorded data. The goal of this project is to develop instrumentation that will allow real-time CSI analyses to be conducted by non-expert users in a fully automated fashion. This project is in support of an explicit need of the Cardiovascular Alterations Research Team to assess alterations in cardiovascular regulation and stability in response to environmental changes (e.g., microgravity). It will support development of countermeasures and then monitor their effectiveness in bed rest studies, in the Space Station, and on planetary missions. Other Teams (Bone Demineralization/Calcium Metabolism Team, Neurovestibular Team and Human Performance Factors, Sleep and Chronobiology Team) may use the technique to measure changes in autonomic nervous function. In addition, this project has many applications to important problems in clinical medicine such the diagnosis and management of patients with diabetic autonomic neuropathy, heart failure and hypertension.

Accomplishments:

- Development of the engineering prototype has been completed.

The CSI was originally developed as discrete acquisition and processing components using readily available clinical instrumentation and processors. A significant part of the current development effort was the design and implementation of a fully integrated engineering prototype for data acquisition and processing. The system has been built around a Thinkmate (Pentium II) computer; standard clinical monitors collect biophysical information and interface with the processor through a Keithly analog-to-digital converter interface.

- A robust user interface has been developed to provide real-time data.

The CSI system hardware is fully supported by a custom GUI-based user interface. The system supports two modes of operation: real-time on-line analysis and off-line post-processing. The

on-line mode supports full-fidelity recording of input data so that it can be re-processed in the off-line mode.

- Application software has been successfully ported to a portable device.

The CSI method of assessing cardiovascular regulation originally required that an expert user conduct off-line post-processing of acquired data. The processing had involved data pre-processing, segment identification, analysis, and interpretation. As a result of the subject effort, the data acquisition and processing pipeline has been fully automated. The user now requires minimal training in order to generate valid results, thus the system is available to a broader user community, including astronauts and other NSBRI investigators.

- CSI utility has been demonstrated on human test subjects.

CSI has been used in clinical studies of autonomic function alterations during induced motion sickness and as a result of diabetic autonomic neuropathy. The system developed under this effort has also been tested in conjunction with NSBRI bed rest studies conducted by the Cardiovascular Alterations Team.

Project 3: Miniature Time-of-Flight Mass Spectrometer
PI: R. S. Potember, Ph.D.

A high-resolution miniature time-of-flight mass spectrometer, already under development for other purposes, has been adapted for space flight. This instrument has the potential to identify and quantify a wide variety of biomarkers to support biomedical research and medical care. It is a rugged device that will unambiguously identify samples containing many compounds and be less than one cubic foot in size, weigh less that 5 kg, and require less than 50 W of power. Its applications include: analysis of breath, body fluids, products of infection, and perhaps DNA repair products and DNA mutations. Identification of compounds with mass ranges from under 100 to more than 10,000 amu has been demonstrated. While the instrument has a wide range of usage, the funds available limited the initial use to analysis of a variety of compounds in fluids although the instrument will be expandable for the other identified uses. As currently configured, this instrument is of special value to the Bone Demineralization/Calcium Metabolism Team and the Muscle Alterations and Atrophy Research Team as well as being useful for gathering data on a variety of other experiments for the other Research Teams.

Accomplishments:

- Engineering model hardware has been successfully implemented.

A major objective of this project was the design and development of a mass spectrometer *system architecture* that can be utilized for diagnostics based on complex, non-volatile biomarkers species. An orthogonal extraction time-of-flight mass spectrometer (TOFMS) analyzer, incorporating a dual matrix-assisted laser desorption/ionization (MALDI) and electron ionization (EI) source, was successfully completed and demonstrated. This novel instrument greatly expands the spectrum of biomarkers that can be measured by incorporating the capability of electron impact ionization with the previously demonstrated MALDI measurements. The device has the potential to evolve into a small (<1 ft^3), lightweight (<5 kg), low power (<50 W), rugged device for continuous operation with advanced signal processing diagnostics.

- Techniques for detection and analysis of urine biomarkers have been successfully developed.

Sampling from urine has been chosen as a high priority for this project. Using the TOFMS, the project team has successfully recorded full spectrum mass spectral signatures of key target biomarker analytes using the MALDI technique at physiological concentrations found in urine. Compounds investigated included: insulin-like growth factors (IGF-I), Urinary 3-methylhistidine, and estradiol. IGF-I is a potent anabolic factor that mimics most of the growth promoting actions of growth hormone (GH) *in vivo*. IGF-1 has also been identified by the Bone Demineralization/Calcium Metabolism Team as an important biomarker. Initial laboratory studies with other bone biomarkers, trivalent hydroxypyridinium crosslinks and creatinine, were completed.

Another biomarker identified to be important to the Muscle Alterations and Atrophy Team is urinary 3-methylhistidine (3-MH). MALDI techniques were applied to *quantitatively* measure 3-MH in biological fluids. Various concentrations of 3-methylhistidine in water and in urine were analyzed to determine the relationship between analyte concentration and analyte molecular ion intensity.

- Techniques for detection and analysis of blood biomarkers have been baselined.

Whole blood is the biological fluid of choice for therapeutic drug monitoring and for performing pharmacokinetic studies. Spectra for whole blood were recorded in DHB matrix and in cyano-4-hydroxycinnamic acid matrix. These spectra exhibited well-defined peaks between 100 to 400 mass units.

- Biomarkers in breath and saliva have been successfully analyzed.

A breath monitoring system was used to examine human subjects in order to select molecules that may serve as biomarkers of normal and abnormal physiology. These molecules will be used to direct the selection of molecules to be monitored with the time of flight miniature mass spectrometer.

The NSBRI Human Performance Factors, Sleep and Chronobiology Team has identified that there is a critical need for in-flight assessment of melatonin levels to develop strategies to monitor the circadian physiology of astronauts during long-duration space missions. Because the sampling of plasma melatonin is an invasive procedure, it would be desirable to have a means of measuring salivary melatonin in subjects on long-duration space missions. A preliminary analysis method of measuring salivary melatonin, using MALDI TOFMS has been developed to provide a reliable, convenient, and economical way to track melatonin during space missions.

- Quantitation of analysis has been successfully demonstrated.

As noted above, 3-methylhistidine in water and in urine was analyzed. The concentrations used in this study were based on 3-methylhistidine (3-MH) concentration typically found in urine, i.e., 20pmole – 3.5nmole. The utility of two types of internal standards, histidine, a structural analogue, and d_3-3-methylhistidine, a stable-isotope labeled analogue were examined. 3-MH samples in water and urine were prepared ranging from 5uM – 10mM, keeping the (3MH)/(histidine) ratio constant at 1:10. Protonated molecular ions for 3MH and histidine could be identified in the corresponding MALDI spectra. The ratio of relative peak intensities of (3MH)/(d_3-3-MH) verses 3-MH concentration gave a linear response with a correlation coefficient, $R^2 = 0.9799$ and a relative standard deviation of the slope of 4.00%.

Project 4: Portable Neutron Spectrometer
PI: R. H. Maurer, Ph.D.

Galactic and solar cosmic rays are inordinately effective at producing secondary neutrons when they encounter spacecraft or habitat material. These neutrons can cause cellular and DNA damage to those exposed. The neutron component of radiation in a space structure is estimated to be between 30 to 60 percent of the total radiation environment when outside the Earth's magnetic field. To be able to measure the neutron spectrum, a portable brief case size, real-time neutron spectrometer prototype with a mass of less than 10 kg has been developed to support the research of the Radiation/DNA Effects Research Team. It can be used to characterize the environment for the development of a countermeasure and also can be used as a real-time monitor to control the application of countermeasures. The instrument will measure neutrons in the range from 10 KeV to 500MeV with at least 10 percent energy resolution and count the number of neutrons below 10 KeV. This portable instrument will incorporate the latest advances in energetic particle detection technology, including energy loss and total energy measurement, while building on the successful charged particle instruments built by JHU/APL for NASA/GSFC and NASA/JPL for many previous near-Earth and planetary missions. The neutron energy spectrum will be measured and an alarm will be incorporated to warn the astronaut when a threshold is exceeded. The devices can be ported within a space vehicle to map the local neutron environment and used similarly on planetary excursions. This project is in support of an explicit need of the Radiation Effects/DNA Damage and Repair Team.

Accomplishments:

- Development of a hardware prototype device has been successfully completed.

The neutron spectrum of interest spans several orders of magnitude from the thermal (keV) to the highly energetic (MeV). There does not exist a single detector technology that will provide adequate sensitivity over this large range. The project team examined a number of detector schemes that might be combined to provide adequate spectrum span with sufficient overlap. Initially, it was thought that three detectors would be required. However, as a result of controlled evaluation and careful design, it was possible to provide the required system performance with two detectors. The project team was successful in developing a hardware prototype device that makes use of a ^{3}He tube, a solid state Si detector, and associated electronics. The use of two detectors, vice three, reduces the size, weight, and complexity of the resultant device.

- Modeling of high energy response has been successful.

Modeling of the response of the high energy channel from detailed cross-sections of the basic neutron-silicon interactions was undertaken using state-of-the-art computer codes. The purposes for developing the models were: to assess the accuracy of these codes for neutron-silicon interactions; to use these codes to understand the results of the energy deposition measurements; to determine whether these codes can be used to calculate the effects of packaging and instrument surroundings on the incident neutron spectrum; and, to assess the ability of the codes to supplement the experimental determination of the instrument response function. A model of the high energy channel was first developed in MCNPX code, a combination of MCNP which is widely used in nuclear engineering and LAHET which is often used in accelerator design and other high energy physics calculations. The model did not reproduce the energy deposition spectra measured at the Columbia University Radiological Research Accelerator Facility (RARAF). Investigations showed that neutron calculations in MCNPX are performed in such a way as to preclude use of this code for energy deposition calculations without a major

restructuring of the code. During this process, GEANT4, a code library widely used in high-energy detector design and simulation produced at CERN, was released. The project team's first model using GEANT4 uncovered several problems with the library, which were quickly addressed by the developers. The current model using GEANT4 reproduces the energy deposition spectra measured at RARAF reasonably well, although discrepancies for the highest energy depositions remain to be resolved. One interesting result of this model is a discrepancy at low energies indicating that gamma production during exposure may not have adequately been accounted for by measuring the background spectrum when the beam is off.

- Demonstration and calibration trials have been conducted at various beam sources.

Device performance has been evaluated and demonstrated at a number of neutron energy levels. The project team conducted experiments at a variety of beam sources and compared the developmental system against qualified reference standards. Beams that were used included the ^{252}Cf and americium-beryllium sources at the National Institute for Standards and Technology (NIST), the plutonium-beryllium source at Clemson University, the Radiological Research Accelerator Facility at Columbia University, and the Los Alamos Neutron Science Center.

- High-altitude aircraft testing has been conducted.

A large part of the GFY 00 effort was directed at the assembly of the integrated hardware prototype system. Before that time, the project developed and evaluated separate low energy and high energy detectors interfaced to laboratory grade support components (i.e., power supplies, amplifiers, processors). Motivation for the development of the integrated system was the opportunity to conduct high altitude aircraft flight tests to acquire performance data in a environment that is richer in neutrons. The integrated hardware prototype included the two system detectors, support electronics, mechanical fixturing, power supplies, and data recording. The device needed to be qualified to the F15/18 vibration, temperature, and pressure environment; a significant amount of environmental testing and design adaptation was performed. The device was flown in April 2000. At 39,000 feet the device experienced corona break-down and subsequent component failure that precluded further testing. Re-design of the integrated device to include a small pressure vessel has been done in anticipation of an additional opportunity to conduct high altitude flights.

- Additional NASA funding was awarded for materials characterization.

The proposed research, "Development of a Neutron Spectrometer to Assess Biological Radiation Damage Behind Spacecraft Materials," was selected for funding for the period May 2000 through November 2003. The primary responsibility under this grant is to support the Lawrence Berkeley Laboratory (LBL) personnel in the evaluation of spacecraft structural and shielding materials by supplying a version of the neutron spectrometer suitable for accelerator tests. The first experiments are scheduled for January 2001 at Brookhaven National Laboratory (BNL). These experiments will collide high-energy heavy ion beams with standard and novel spacecraft materials and the spectrometer will measure the neutron energy spectrum produced as a result of these collisions. A secondary responsibility for this grant is to continue development of the modeling effort in a manner that is useful for materials science experiments as well as for assessment of astronaut biological radiation risk. Success using the GEANT4 Monte Carlo code in modeling the neutron silicon interactions will supplement the current NASA modeling efforts which employ deterministic Boltzmann transport and FLUKA Monte Carlo approaches.

- Additional funding awarded for experiments on a Mars Rover mission

The proposal research, "MArtian Neutron Energy Spectrometer (MANES) for the Mars 2003 Lander Mission," was selected, in November 1999, for a stage of further definition as a potential instrument on the Mars 2003 Lander. The purpose is to determine the neutron background levels to assess astronaut safety. Due to subsequent cancellation of the Mars 2003 "large" Lander, the instrument is on hold until a decision is made about a Mars 2005 Lander. An extended definition phase grant, to study various instrument configuration options as contingencies for future mission concepts, has been received. The project team supported a Mars 2003 Lander payload accommodation meeting in Pasadena on December 7-9, 1999.

Due to the failures of the September and December 1999 Mars' missions the whole Mars Surveyor program was put on hold at the end of January 2000. Subsequently, NASA decided to fly only an Orbiter in 2001 (April 2000 decision) and two smaller Athena Rover/Landers in landing bags in 2003 (July 2000 decision). The first decision and anticipation of it by NASA JSC resulted in a request to consider the option of combining Dr. Gautam Badhwar's Mars 2001 Lander MARIE instrument with the MANES instrument for a possible large Mars 2003 Lander. This combination was called MARINES. A technical interchange meeting was held at JSC on March 1-2, 2000 and a formal presentation was made on both MANES and MARINES at NASA Headquarters on March 29, 2000. The July 2000 decision to fly the two Athena Rovers in 2003 has resulted in any future JSC funding being put on hold for MANES until NASA makes a decision about the Mars 2005 mission.

General team accomplishments:

The NSBRI Technology Development Team is characterized as an integrated, multidisciplinary group chartered to develop systems, instrumentation, devices, and algorithms. The accomplishments noted above provide a clear demonstration that this objective has been achieved. In addition to this, the project teams have demonstrated unique capabilities of being able to structure and accomplish complex applied research and development. Some of the characteristics that cross project boundaries are:

- The capability to successfully conduct rapid system prototyping.

All of the Technology Development Team projects were successful in accomplishing the goal of developing and demonstrating prototype system implementations. A number of patent disclosures and/or applications have resulted from the developments. The ability to support the development of practical and useful tools in support of basic research requirements is a necessary element of a successful undertaking such as the NSBRI.

- The capability to transition developments to practical embodiments.

As an extension of the prior item, it is not sufficient to develop unique, one-of-a-kind prototypes. The developments must have practical means of supporting the basic research efforts by providing reliable and robust tools. The Technology Development Team projects have successfully demonstrated the ability to transition their developments to the real-world environment. For example, the Instrumentation for Assessment of Cardiovascular Regulation has been successfully used in bed-rest studies and the DEXA Scanner is ready to support human subject research studies. As well, some of the developments have been shown to be commercially viable for the Earth-based market.

- The capability to network and collaborate with NASA, the medical community, etc.

All of the Technology Development Team projects have established close and on-going interactions with NASA and the medical community. The interactions were initiated during the project proposal phase to assure that the intended development addressed a current space issue and was founded in a practical medical basis. The interactions have experienced positive growth and expansion throughout the research and development cycle. The result of the networking is that the resultant development products have validated utility to the space and medical communities. And, the networking within the communities has provided very good exposure and visibility for other applications and opportunities.

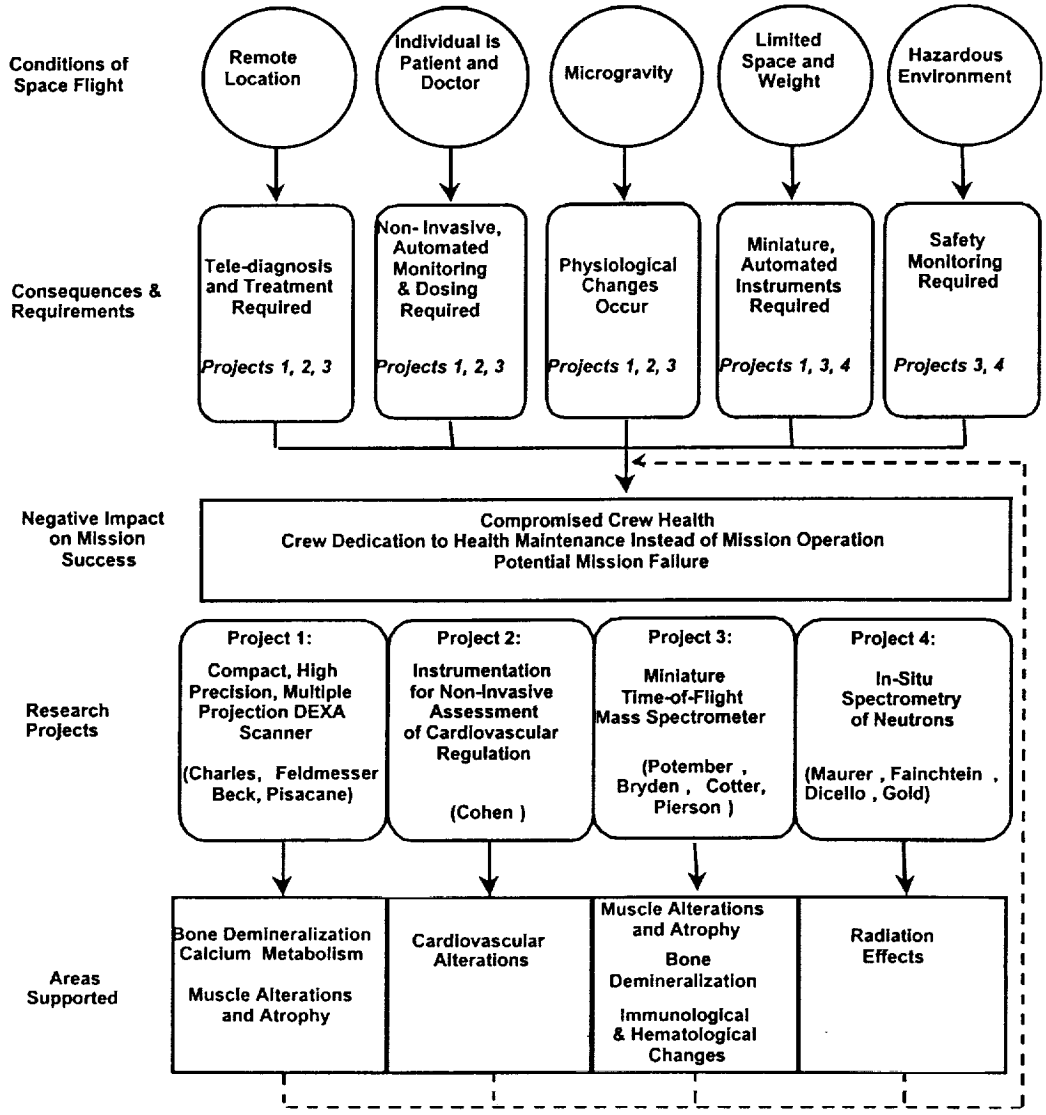
- The capability to produce quantifiable results to support countermeasures research.

The basic research programs of the NSBRI are charged with developing and evaluating countermeasures to the effects of long endurance exposure to microgravity. This effort requires that cause and effect relationships be identified and characterized. Proper characterization mandates that empirical data be referenced to a standard and be quantitative in nature. All of the Technology Development Team projects have achieved a level of standardization and quantitation that is necessary to support the basic research initiatives. In fact, some of the engineering models that have resulted from the team's activities exceed the accuracy and precision found in existing clinical and commercial systems.

II. RISK REDUCTION ACHIEVED BY PROGRAM

The risks associated with long-term exposure to microgravity and a high radiation environment are numerous; they represent the basis for the research program pursued by the National Space Biomedical Research Institute. Most of the ongoing NSBRI research is vertically integrated within a specific thrust area. For instance, the Research Teams typically have a core research topic that is combined with several special topic areas to form a disciplined approach to addressing a number of related issues.

The Technology Development thrust area is implemented in a different manner. The funded projects are selected, among other reasons, for their ability to provide necessary and enabling technologies for the basic research areas. Thus, the thrust area is laterally integrated with the other research areas. Figure 1 is the process flow diagram that describes the issues and associations for the Technology Development projects funded during GFY 98-00. The noted support areas indicate the lateral integration.



Section I provides a detailed account of the Technology Development Team projects, including the accomplishments and the focus of the basic research areas that are supported. The balance of this section addresses the risk reduction that was achieved by the Technology Development program in terms of the supported basic research areas.

Bone Demineralization Research Risk Reduction

Two challenges associated with the development of countermeasures for bone demineralization include the understanding of the mineral loss process and being able to monitor the instantaneous condition of the subject's bones. Technology Development project 3 specifically addresses the process issue, while project 1 specifically address the monitor issue. Both projects have completed engineering model developments that have demonstrated the ability to provide quantitative information that is critical to the current and future research of the Bone Demineralization Team.

The devices have been designed to be directly adaptable for in-space use. Size, weight, and power are currently, or will soon be, appropriate for routine launch and regular use on-orbit or in missions beyond Earth. The devices and their associated methods are highly automated. The intended users for the devices are people with limited skills and no special training requirements. Thus, the devices have broad utility in both space and Earth-based applications.

Historical space-based monitoring techniques have typically relied on a method of specimen sample-and-storage. Specimen analysis may, under good conditions, be completed many months after completion of the mission. This retrospective assessment may provide a limited capability to understand processes, but it certainly does not afford the ability to provide closed-loop monitoring and control of countermeasures. Both projects 1 and 3 generate data that are available in (near) real-time, thereby providing a means to achieve the closed-loop. A secondary benefit accrues by virtue of not requiring the volume and utilities associated with specimen storage facilities.

The bone demineralization conditions that astronauts experience are similar to those that exist in clinical populations (e.g., advanced age, quadriplegia) on Earth. Thus, the research that will be supported by projects 1 and 3 is expected to have a direct positive influence on a much broader population than just the astronaut community. As well, the technology itself has demonstrated better performance than commercially available devices; therefore, clinical versions of the technology will prove useful in the general clinical environment.

Muscle Alteration Research Risk Reduction

Muscle alteration research faces the same challenges noted, above, for bone demineralization. Technology Development projects 1 and 3 provide the same armament of tools in support risk reduction for muscle as they do for bone. At this point in time, though, the project 1 performance is less well developed for muscle than it is for bone.

Cardiovascular Alterations Research Risk Reduction

Orthostatic intolerance can result in syncope when an individual is subjected to gravitational influence after exposure to microgravity. This situation can pose severe risks to astronauts who have to execute unassisted emergency procedures or extraterrestrial landings. The ability to predict, prevent, or control orthostatic intolerance and its effects is significant to the space program. Project 2 has adapted hardware and a cardiovascular system identification

application into a self-contained, automated device for measuring and characterizing alterations in cardiovascular regulation. Application of the resultant device will play a critical part in understanding the mechanism of orthostatic intolerance, developing suitable countermeasures, and assuring that the countermeasures are fully effective while on mission. The device will also be useful for clinical diagnosis and treatment of patient populations suffering from cardiovascular and neurological disorders caused by a variety of medical and environmental factors.

Radiation Effect Research Risk Reduction

Exposure to radiation in space is a threat that can lead to an increased risk of cancer and DNA damage. A significant portion of the exposure, between 30-60%, results from neutron sources which are extremely difficult to monitor, let alone characterize, in real-time. Absence of a portable, quantitative, real-time neutron spectrometer results in an exposure safety risk for astronauts. Project 4 was proposed to develop such a neutron spectrometer and supply information on the neutron environment to the Radiation Effects Team in support of assessing radiation damage and cancer risk.

The project team was successful in developing an engineering model of a neutron spectrometer. The device, when fully configured for flight operations, is projected to weigh less than 10 kg and provide real-time, quantitative spectral energy data over the range of 10 keV to 500 MeV. The device has been validated in various energy-level neutron beams, and has been flown in a high-altitude aircraft test. System performance models have been developed to characterize the device transfer function. And, the device has been selected for further development by NASA to support materials characterization studies as well as future unmanned MARS missions.

Chronobiology Research Area

While not originally a focus of the Miniature Time-of-Flight Mass Spectrometer project, the development is capable of supporting the research of the Human Performance Factors, Sleep and Chronobiology Team by measuring melatonin in saliva as opposed to the invasive techniques now used.

During GFY 99, researchers on one of the NSBRI Human Performance Factors, Sleep and Chronobiology Team projects identified the need to collect blood samples from test subjects at about 15-minute intervals over a 24-hour period. The researchers had identified a catheter that would provide suitable service, but they were unable to locate an appropriate sample pump and distribution system. Process automation was necessary to efficiently and cost-effectively support research associated with circadian rhythm.

The Technology Development Team examined a number of technology alternatives to satisfy the unmet needs. In April 1999, the Technology Development Team hosted a workshop of the Human Performance Factors, Sleep and Chronobiology Team researchers and technical personnel to discuss the research needs and issues. A design and development strategy was defined at the meeting. Subsequent action on the strategy was undertaken by JHU/APL under its own internal funds.

Other Research Areas

In the first three years of funded activity, the Technology Development Team did not have any directly funded projects that specifically addressed risk reduction in the other NSBRI

basic research areas. That did not, however, preclude the team from evaluating the needs and requirements of those areas as noted in the items that follow.

- Technology Development Working Group

The identification of critical technology needs for the NSBRI is an evolving process. To assure that the current and emerging technology development needs of the Institute Research Teams are met, the Technology Development Team has constituted a Working Group. The Working Group (Table 1) is composed of representatives from each of the NSBRI Research Teams. One of the Working Group activities has been the development of a document, the NSBRI Technology Development Requirements. The requirements document contains materials that are provided in order to solicit commercial and/or developmental solutions to the specific research needs. The document was originally issued in January 1998 at the first NSBRI retreat. The latest version was issued in April 1999; working updates to the document have been maintained since 1999.

- Workshop on Technology Development

In support of the NSBRI call for proposals for a GFY 01 start, the Technology Development Team conducted a Workshop on Technology Development in December 1999. The workshop participants included representatives from the eleven NSBRI basic Research Teams, new NSBRI consortium members, NASA, and industry. The outcome of the workshop was that eight interrelated themes were identified that summarized the current and projected technology thrusts for technology development in support of the biomedical aspects of space exploration.

- Presentations and Publications

The Technology Development Team actively supported education and public outreach objectives through its presentations and publications.

Future Programmatic Implications.

The success of the Technology Development Research Team in working closely with the other Research Teams and in developing important systems that meet their needs validates the utility of this program to the NSBRI. Because of its nature of development as opposed to research and the unique inter-team interests, the Technology Development Research program should be evaluated with different criteria. For example, the inter-team interactions are as significant if not more that the intra-team interactions. Development of instrumentation systems is not inexpensive and each of the developers were faced with severe financial constraints that limited their accomplishments. Except in software intensive projects, component costs generally consume a significant amount of the available funding leaving less than desired for labor. In many cases, the research utility of the systems will be fully demonstrated by follow-on activities not covered in this development phase, which is decidedly different from the research carried out by the more traditional Research Teams.

Members of the Technology Development Working Group

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Cardiovascular Alterations	D. Sherman, Ph.D.	MIT
Muscle Alteration and Atrophy	R. Schwartz, Ph.D.	BCM
Bone Demineralization/Calcium Metabolism	T. Beck, Ph.D.	JHU
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Technology Development Working Group Leader	V. Pisacane, Ph.D.	JHU

Table 1

ADVANCED MULTIPLE PROJECTION DUAL ENERGY X-RAY ABSORPTIOMETRY (AMPDXA) SCANNING SYSTEM

(Formerly: Compact, High Precision, Multiple Projection DEXA Scanner)

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ADVANCED MULTIPLE PROJECTION DUAL ENERGY X-RAY ABSORPTIOMETRY (AMPDXA) SCANNING SYSTEM

EXECUTIVE SUMMARY

The purpose of the Advanced Multiple Projection Dual Energy X-ray Absorptiometry (AMPDXA) Scanning System project is to design, build, and test a precision scanner system for monitoring the deleterious effects of weightlessness on the human musculoskeletal system during prolonged spaceflight. The instrument uses dual energy X-ray absorptiometry (DXA) principles and is designed to measure bone mineral density (BMD), decompose soft tissue into fat and muscle, and derive structural properties (cross-sections, moments of inertia). Such data permits assessment of microgravity effects on bone and muscle and the associated fracture risk upon returning to planetary gravity levels. Multiple projections, coupled with axial translation, provide three-dimensional (3-D) geometric properties suitable for accurate structural analysis. This structural analysis coupled with bone models and estimated loads defines the fracture risk. The scanner will be designed to minimize volume and mass (46 kg goal), while maintaining the required mechanical stability for high-precision measurement. The AMPDXA will be able to detect 1% changes in bone mass and geometry and 5% changes in muscle mass.

The development of the AMPDXA is being carried out in a multistage process beginning with an initial Laboratory Test Bed to prove basic principles and develop initial hardware and software. Following the Laboratory Test Bed was a Clinical Test System, which is now operational, which will allow the testing of AMPDXA principles on human subjects as well as on special phantoms and other test objects. The third stage is the design and development of a protoflight system that incorporates all the design and performance features of the previous units into a form, fit and functional configuration that could evolve into the low mass flight unit. Preliminary designs for this protoflight system incorporate advanced electronic fabrication technologies (chip-on-board, multichip modules) coupled with commercial (off-the-shelf) parts to produce a reliable, integrated system which not only minimizes size and weight, but because of its relative simplicity, is also cost effective to build and maintain. Additionally, the protoflight system is being designed to minimize power consumption. Methods of heat dissipation and mechanical stowage (for the unit when not in use) are being optimized for the space environment. The latest concept for the protoflight AMPDXA system is shown in Figure 1.

The AMPDXA Project is a joint effort between the NSBRI's Technology Development Team and both the Bone Demineralization/Calcium Metabolism Team and the Muscle Alterations and Atrophy Team. Its goal is to provide the high precision monitoring system necessary to fully assess both the deleterious effects of weightlessness on the bones and muscles and the effectiveness of any countermeasures. We believe that any pharmacological or exercise-related countermeasures used by astronauts to mitigate microgravity effects will require efficient and timely monitoring. Moreover, the monitoring device must be capable of being used by astronauts during spaceflight so that feedback can be dynamically employed to regulate countermeasure doses. The system design will be such that intelligent but not necessarily medically trained personnel will be able to create scans that will provide all of the accuracy and

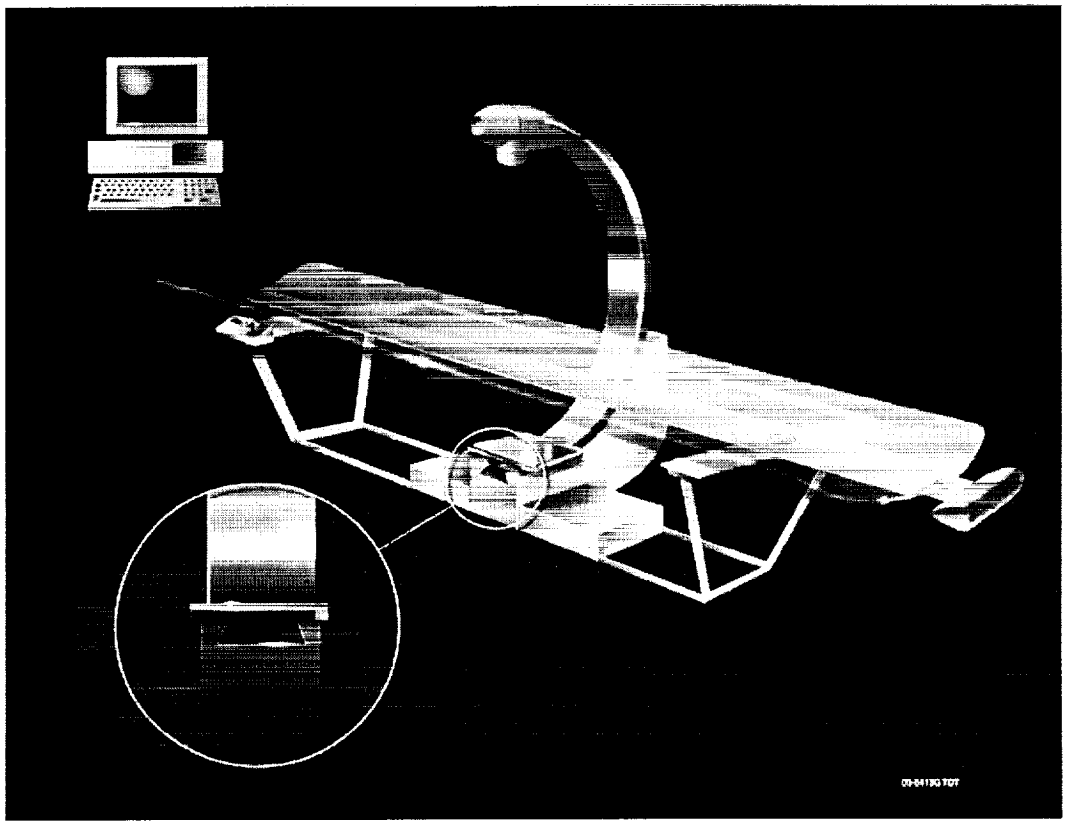


Figure 1. Artist's concept of space-based protoflight AMPDXA.

precision necessary. Readouts and displays for the AMPDXA instrumentation will be specifically designed to provide useful (real-time) feedback information to both the astronauts and the ground-based physician monitoring team (as permitted by the mission dynamics).

We believe the key to understanding the mechanism of bone (and muscle) loss in space (microgravity) lies in the bone's structural details and the changes in the structure due to prolonged weightlessness. Our hypothesis is that throughout most of adult life, aging bones become more structurally efficient and retain their strength even though BMD declines. The homeostatic mechanism for strength maintenance depends on skeletal loading. Thus, to maintain bone strength, normal loading on the skeletal system must be maintained. Absence of loading during prolonged spaceflight (or disuse) can cause uncompensated loss of bone strength. Even reduced loading (caused by muscle wasting and inactivity in the elderly) can cause a disruption in the bone strength maintenance mechanism.

Current bone and muscle mass measurements (via conventional DXA or ultrasound) are regional averages that obscure structural details. Since the mechanical consequences of lost bone and muscle are reflected in the structure, an absolute determination of skeletal mechanical competence is needed to supplement the loss measurements. Engineering properties of the bones can be derived from DXA-generated BMD data. Our method derives geometrical measurements

from the BMD images. From such images, we extract BMD profiles at important skeletal locations (e.g., proximal shaft and femoral neck). Key properties measured and derived from these profiles include the BMD, the subperiosteal width, the section modulus (related to strength), and the cortical dimensions.

During the course of the AMPDXA Project, significant progress has been made in several key areas: (1) instrument development, (2) algorithm development for BMD image extraction and structural analysis, and (3) bone reconstruction and modeling techniques. As mentioned above, a full size Laboratory Test Bed (1 meter source to detector distance) was constructed to verify principles and theoretical predictions. Scanning is provided by high-precision rotation and translating stages. This Laboratory Test Bed, in conjunction with a high-resolution detector and our analysis software, has produced some exciting preliminary results. Figure 2(a) is a BMD image of a human femur immersed in a cylinder of water (simulates fatty tissue). The same bone was imaged on a new commercial DXA scanner located at the Johns Hopkins Hospital as shown in Figure 2(b). The improvement in spatial and contrast resolution with our scanner is quite evident by comparing the two figures. This improvement is further elucidated by the graph in Figure 2(c). The curves are measured bone projected thicknesses on a slice through the femoral shaft. The fine variations on the AMPDXA profile are not noise, but reflect small changes in the actual bone thickness.

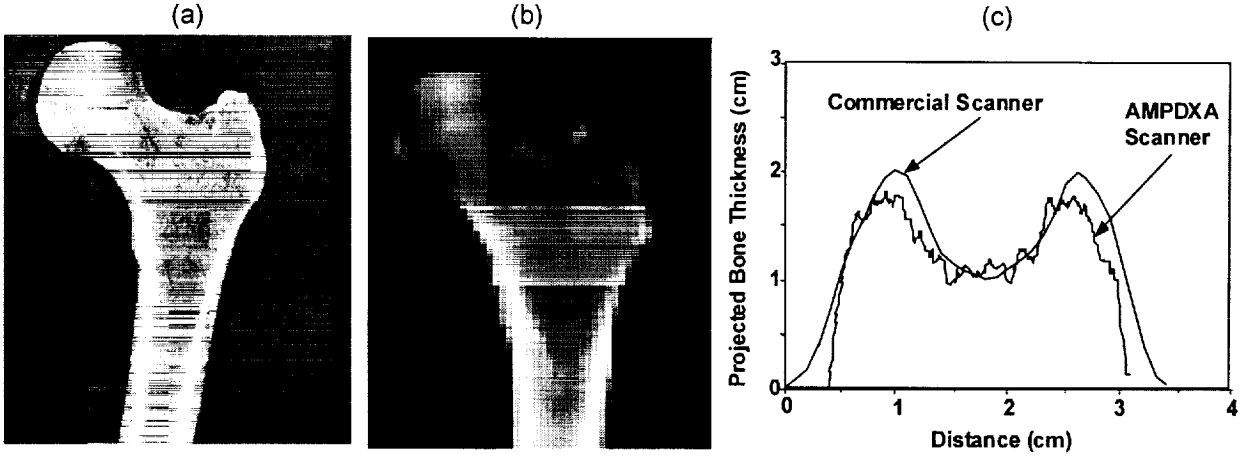


Figure 2. Comparison AMPDXA versus conventional DXA. (a) AMPDXA BMD image. (b) Commercial DXA BMD image (same bone as (a)). (c) Bone mass profiles (AMPDXA and commercial) with distance across a given bone section..

The next important instrumentation step has been the development of a Clinical Test System. The Clinical Test System incorporates a high precision rotation and translation stage to provide the scanning capability to carry out qualification tests on human subjects. Since the Clinical Test System is designed to operate only on earth, the table and gantry were not built to the size and mass requirements of a protoflight AMPDXA. In fact, the unit was built on a used CT Scanner structure (Figures 3 and 4). Employing used equipment for some of the structural elements and the rotating parts and machinery has allowed critical resources to be focused on the information extraction and analysis issues leading to human testing.

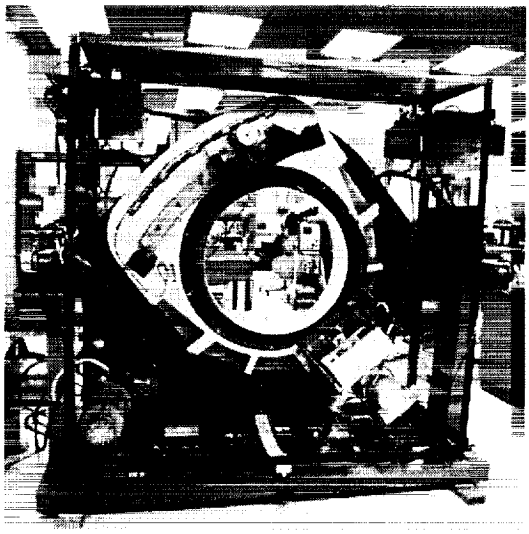


Figure 3. Clinical Test System gantry showing x-ray tube with collimator and filters and movable detector.

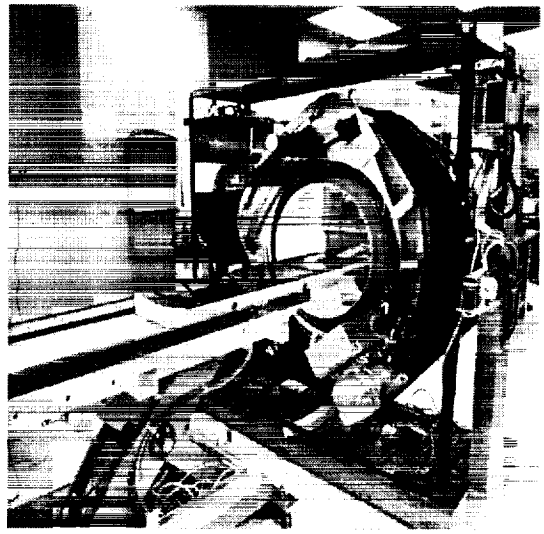


Figure 4. Clinical Test System with couch.

The image extraction capability of the AMPDXA is illustrated above in Figure 2, where not only is the BMD image higher resolution, but also the mass distribution in a projected thickness of a femur slice contains much more structural detail than conventional DXA's (see Figure 2(c)). The high frequency content of the BMD spatial projections as shown in Figure 2(c) are reproducible and provide information on the bone's microstructure. Work on multiple image analysis has progressed quite well as shown in Figure 5, which illustrates BMD images of the same bone collected in seven different orientations. Using multiple projections about the bone axis allows structural properties (e.g., bending strength) to be obtained independent of patient position. To do this at least three arbitrary projections over 90 degrees (two of which are orthogonal) must be obtained. Such analysis can provide maximum and minimum moments of inertia for bending or torsion in any plane. Our experiments to date with different sets of three projections show that the principal moments of inertia can be determined within 3 to 4%. Additional projections (above 3) reduce this number further. Our experimental systems also have some known non-linearities which when removed will drop the error in the three projection estimation of moments to less than 1%.

Initial multiple projection work also shows that three projections are not sufficient for total image reconstruction; however, it appears that a cone beam type reconstruction from as few as three to seven projections is sufficient to produce a pseudo three-dimensional geometry that is mechanically equivalent to the measured hip. A seven-projection, cone-beam reconstruction is shown in Figure 6.

The AMPDXA, as described above, has direct application to risk reduction in NASA's Critical Research Path. The AMPDXA is capable of real-time monitoring of bone and muscle loss at extremely high precision. Since the results are patient-specific and not tied to volumetric

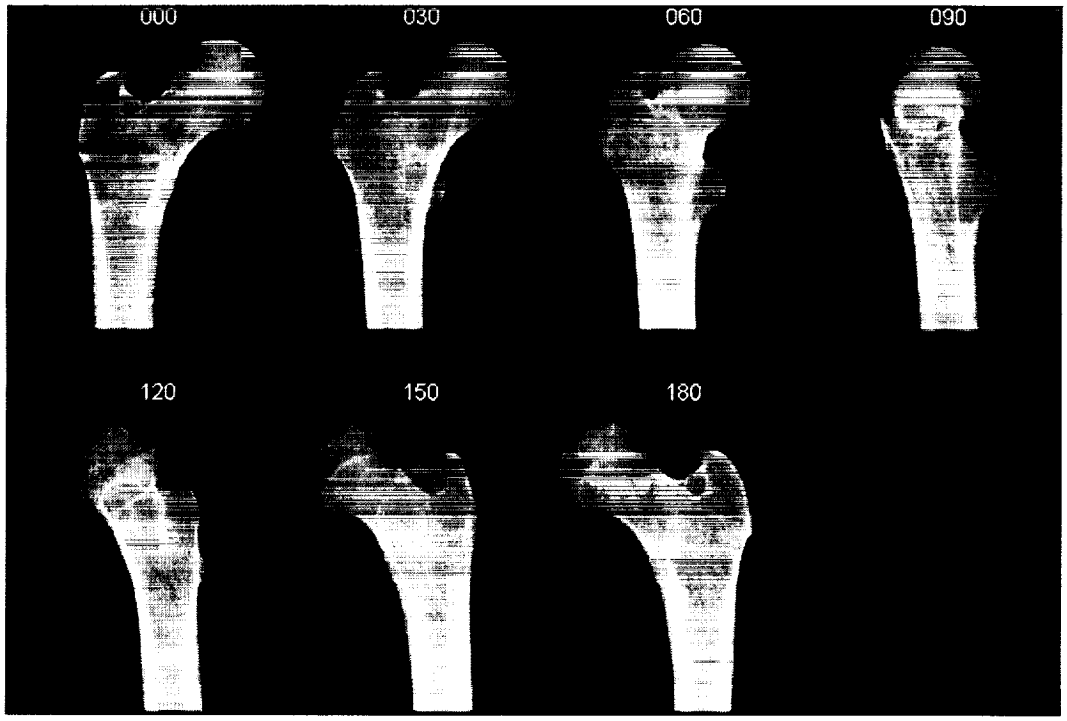


Figure 5. Multiple DXA projections using rotational stage.

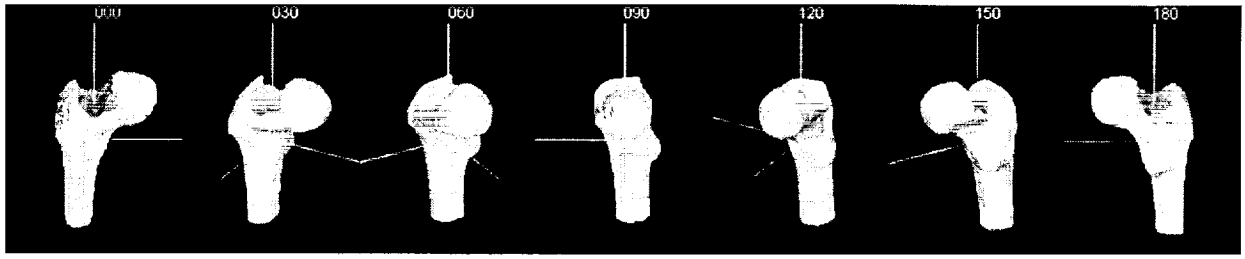


Figure 6. Cone-beam reconstruction from minimum projection data. Seven projections were utilized in this unfiltered reconstruction.

averages and statistical norms, the AMPDXA is a very useful tool for monitoring the effectiveness of countermeasures as well as determining the risk of fracture under deployment scenarios. The AMPDXA also appears to be a natural adjunct to earth-bound research on the effects of aging and disuse on bone integrity. It could also be used as a routine screening tool for osteoporosis and as a monitoring tool for osteoporosis drug therapy.

ADVANCED MULTIPLE PROJECTION DUAL ENERGY X-RAY ABSORPTIOMETRY (AMPDXA) SCANNING SYSTEM

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ADVANCED MULTIPLE PROJECTION DUAL ENERGY X-RAY ABSORPTIOMETRY (AMPDXA) SCANNING SYSTEM

I. PROJECT RESEARCH ACTIVITY

A. OBJECTIVES AND SPECIFIC AIMS

A.1 Need

An important NASA Critical Research Path concern for future long-term spaceflight is bone loss and muscle atrophy from prolonged exposure to microgravity [1]. The magnitudes of tissue loss can be alarmingly high and may severely impact the performance of astronauts upon return to gravitational fields. Inability to perform activities due to weakened muscle and fragile bones is problematic in returning to Earth, but possibly life threatening if occurring on a remote planet with limited medical resources. With restricted access to full medical treatment during long duration spaceflight, bone fracture may prove to be catastrophic, especially because healing rates in the absence of mechanical stimulus (load) are believed to be degraded [2]. Of equal concern is the possibility of major injury to an astronaut's musculoskeletal system in an emergency action (rapid egress following a water landing, for example) after a prolonged stay in orbit. Experience with bed-rest subjects, astronauts and cosmonauts indicates that the magnitudes and patterns of tissue loss are extremely variable from one individual to the next, and also between different body regions. Little mass appears to be lost from the upper extremities during weightlessness; whereas the rate of mass loss from the vertebrae, pelvis, and proximal femurs of astronauts average between 1.0% and 1.6% per month. This loss should be contrasted to the loss from these sites of approximately 0.8 to 1.3% per year in postmenopausal women [3], which is highly associated with increased bone fragility.

The system of bones and skeletal muscles provides structure to the body and provides the capability to carry out activities. Bone provides the basic structural integrity of the body that carries forces and furnishes a framework for muscle. Recent evidence shows that there are important differences between the way that bone is lost in aging on earth compared to changes observed in spaceflight. On earth, the skeleton is continually loaded during normal activities. Load causes mechanical strains within the bone, which tend to be greatest on the subperiosteal surface. The normal turnover of bone accompanying the aging process causes some net loss of bone from endocortical and internal surfaces. Net loss under loading causes skeletal strains to increase, but in long bones the increase is greater on the subperiosteal surface, not at the internal surfaces where the loss occurred. The result is that new bone is formed on the subperiosteal surface sufficient to maintain the section modulus. Because it takes less new bone on the subperiosteal surface to compensate for bone loss from internal surfaces, strength can be maintained in the presence of net bone loss [4]. This mechanism requires that the skeleton be loaded, but, except for exercise countermeasures, load is absent on the lower skeleton during spaceflight. Not only does bone loss accelerate under diminishing loading, but evidence from Cosmonaut data on Mir suggest that the compensatory changes are absent as well. This means that astronauts may be at a greater risk of fracture for the same loss of bone mass [5]. Thus, it is

important to not only determine bone mass and muscle atrophy, but also to determine the geometrical configuration of the bone structure. From this, analyses unique to specific astronauts can be carried out to estimate the specific load-bearing capability of their musculoskeletal system and forewarn of any danger.

During prolonged spaceflight of months to years in duration it would therefore be essential that astronauts be provided with a quantitative means to monitor tissue loss and structure, gauge risk of serious consequences, and assess the effectiveness of the countermeasures. In the initial space station trials, the AMPDXA scanner would be used to ascertain the efficiency (efficacy) of the various bone and muscle loss countermeasures (i.e., pharmacological agents, exercise, mechanical or electrical stimulation, etc.). Then, during very long orbital flights or interplanetary missions, the AMPDXA would monitor the effectiveness of the chosen countermeasure(s) as well as establish activity risk factors and help diagnose disease and injury.

A.2 Objectives

What we currently know indicates that such a device should be capable of the following:

- Measure both bone and muscle mass at any anatomical location, but in particular, the weight bearing regions of the spine, hips, and lower extremities.
- Measure with a precision sufficient to detect a 1% change in bone mass or a 5% change in muscle mass with 95% certainty from a single measurement.
- Provide accurate assessment of bone structural changes.
- Provide information that would permit the assessment of the consequences of tissue loss (muscle or bone strength) at a given anatomical location (e.g., risk of fracture).
- Allow near real-time access to measurements of bone mass loss, structure, and risk of fracture.
- Provide normal radiographs for the diagnosis of disease and injury.

Any such instrument must also meet certain environmental, safety, volume, and weight requirements for spaceflight and must achieve intended performance levels with little user training.

A.3 Approach

There are several methods for determining bone mineral density (BMD), bone structure, and soft tissue components including computed tomography (CT), magnetic resonance imaging (MRI), ultrasound (US), and dual energy x-ray absorptiometry (DXA). While CT can image and

measure the geometrical characteristics of bone and soft tissue [6], it is not particularly suited for space usage because of its high radiation dose per scan and total body CTs are large and extremely massive (thousands of pounds). MRI, while excellent for imaging soft tissues, suffers from a similar size and weight disadvantage and whole body MRIs consume significant power, generate large magnetic fields, and weigh tens of thousands of pounds.

Thus, the two most promising technologies for adaptation to a spacebase bone and muscle monitoring system are ultrasound and dual energy x-ray absorptiometry. We have chosen to build a scanning system based on DXA principles for the following reasons: While ultrasound systems can be small and lightweight with low energy consumption, physical limitations of ultrasound propagation in human tissue restrict US to measurements of superficial bone (e.g., calcaneus) or thin body parts. Basic principles indicate that reliable ultrasound measurement capabilities in the critical locations of the spine, pelvis and hip are unlikely to be devised. The use of statistical inference to associate measurements at accessible locations (e.g., calcaneus) to critical sites does not appear to be a viable option given the large variation in regional response to bed-rest and microgravity. Ultrasonic methods have not shown the capability of measuring bone geometry and thus present difficult challenges in interpretation of biomechanical consequences of altered bone mass. Finally ultrasonic instruments have not been successfully used for the quantification of muscle mass. DXA, on the other hand, can be used to measure bone in any anatomical location, and is an established standard for quantification of body composition, including muscle and fat mass.

Adaptation of a whole body-capable DXA scanner for space use introduces significant challenges. Commercial devices are relatively heavy, energy hungry and bulky. The fact that DXA employs ionizing radiation requires a careful design of the source and detector system to ensure that radiation doses impose a negligible radiation risk to astronauts during use. Current DXA scanners have not yet demonstrated the level of measurement accuracy and precision which we have specified as desirable for this application. Current scanners also are poorly suited to the measurement of structural details necessary to detect the subtle dimensional changes that underlie strength changes associated with bone loss. None of the commercial units provide an estimation of injury risk due to changes in bone mass and structure.

A.4 Specific Aims

The AMPDXA under development represents a logical evolution of current DXA scanning technology; extending the capabilities in several ways. First, the scanner will be designed not only to measure conventional bone mass and to decompose soft tissue into measurements of fat and lean mass, but to use those measurements to derive three dimensional structural properties. The derived properties include the principle moments of inertia and section moduli as well as dimensions of the cortical bone that bears most of the mechanical stress under load. Accurate measurement of bone dimensions requires higher spatial resolution than existing DXA systems. Resolution of our Laboratory Test Bed is on the order 3.8 line pairs/mm (lp/mm), compared to the 0.1 to 1.0 lp/mm of conventional DXA scanners. The measured geometry will be employed in a three dimensional engineering model of the measured bone to provide the astronaut an assessment of the biomechanical consequences of a given change in bone mass, i.e.,

how the bone strength has been altered. Secondly, the multiple projection technology will permit improvement in measurement uncertainty due to variations in positioning thus reducing user variability as a source of imprecision. Theoretical simulations also indicate that multiple projection data can be used to eliminate a fundamental uncertainty in soft tissue composition, when discriminating bone from soft tissue. Our dual energy decomposition algorithm uses the ensemble of image pairs to quantify tissues in the scanned value rather than decomposing each projection individually. The scanner design permits varying the acquisition time to achieve a constant average signal-to-noise ratio (SNR) at the detector over a wide range of tissue thicknesses. Current scanners use a constant x-ray fluence optimized for a single thickness, thus perform poorly in thicker regions. In our system, a three-element scintillator diode detector is used with tissue calibration materials to monitor the x-ray fluence and any fluctuations in the spectrum that would influence calibration. These latter enhancements result in a significant improvement in precision compared to existing systems or alternative technologies.

There is a growing understanding that changes in bone throughout life are at least partially mediated by local mechanical forces on the bone. Moreover, these forces are dominated by muscle action. Gravitational unloading of the weight-bearing skeleton severely attenuates those forces resulting in rapid loss of muscle mass, which is followed by a slower more insidious loss of bone. There is increasing evidence that a similar process of muscle wasting, followed by changes in bone, is characteristic of the changes associated with osteoporotic fracture in the elderly. This interplay between bone and muscle loss is poorly understood; hence a high precision measurement tool for measuring of both bone and muscle should permit the advancement of knowledge in the study of the consequences of both microgravity and osteoporotic tissue changes.

The AMPDXA Project is a joint effort between the NSBRI's Technology Development Team and both the Bone Demineralization/Calcium Metabolism Team and the Muscle Alterations and Atrophy Team. The AMPDXA Scanner System will provide the high precision monitoring system necessary to fully assess both the deleterious effects of weightlessness on the bones and muscles and the effectiveness of any countermeasures. We believe that any pharmacological or exercise-related countermeasures used by astronauts to mitigate microgravity effects will require efficient and timely monitoring. Moreover, the monitoring device must be capable of being used by astronauts during spaceflight so that feedback can be dynamically employed to control countermeasure dosimetry. Readouts and displays for the AMPDXA instrumentation will be specifically designed to provide useful (real-time) feedback information to both the astronauts and the ground-based physician monitoring team (as permitted by the mission dynamics).

B. RESEARCH MODIFICATIONS

The only significant modifications to our original research proposal are:

(1) *Reduction in weight goals*: A lightening of the projected protoflight instrument weight from a preliminary 200 kg goal to a current target of less than 100 kg, with a projected long-term goal of less than 46 kg (100 lb AMPDXA). The projected

weight budgets for the components of the protoflight AMPDXA are shown in Table 1. These changes are made possible by the continuing evolution of instrument design (see (5) below), improvements (both current and projected) in tube technology, and further refinements in operational scenarios.

Table 1. AMPDXA Weight Budget Estimates and Projections

Item	Projected Weight, kg		
	Current	1-3 Years	3-5 Years
Detector	12	8	6
Detector Electronics	8	6	4
X-ray Tube	22	10	6
High-Voltage Power Supply	14	10	8
Rotating Structure and Mechanisms	13	11	9
Stationary Structures	9	9	9
Table and Drive Mechanisms	8	6	4
Totals	86	60	46

(2) *Change in detector technology*: The original proposal relied upon custom-built diode detectors. These appeared to offer the best possible quantum detection efficiency and total conversion efficiency from x-rays to electronic signal level. Now there are several other detector technologies that can have better conversion efficiencies and they will allow us to use much more of the x-ray tube's output. We focused our attention on large area detectors because more of the tube's output is used for each exposure, thus requiring fewer exposures to cover a large body area. We examined several different scintillator materials with the photodiodes, a large array CCD imager with several phosphor screens, and an amorphous silicon detector array that is approximately 8" by 10". The large area, amorphous silicon detector was selected, no only for its resolution, but also for its anticipated size growth from 8" x 10" to approximately 17" x 17". The current high-resolution detector has 127 μm square pixels distributed in a 1408 x 1888 active array. We have analyzed several phosphor screen materials for use with this array. The current choice is a cesium iodide screen.

(3) *Changes in scanning motions*: The original design used a two-row detector array linearly translated along the patient axis during acquisition. This motion would have to be repeated after energy switching for each projection. Utilizing the amorphous silicon detector removes the necessity of scan motion during image acquisition. The current test systems are able to image an 8" x 10" area without motion, the space-based design may use a larger array on the order of 17" x 17". Required motions for image acquisition will still involve rotation to achieve different projections, but the simplification of the mechanics will assist in meeting weight goals.

(4) *Refinement of x-ray spectra*: The choices of x-ray spectra and beam filters were modified from the original design from 90 kVp (Ho filter) and 120 kVp (Ho+Ce+Cu filters) to 80 kVp (Mo+W filters) and 140 kVp (Gd+Mo+Cu filters). This was done to further improve signal characteristics, radiation dose efficiency, and x-ray tube power efficiency under thick body part scanning conditions, where demands are most stringent.

(5) *Geometry refinement*: Originally a mechanical design using a linear scintillator/ photodiode detector array was proposed that operated on a ring-like gantry structure to allow 360° rotation. As the linear array detector was replaced by the large, flat panel and the drive to reduce both the size and weight of the entire unit became a major focus, the ring gantry was replaced by a "C"-arm gantry as shown in the artist's rendering in Figure 1.

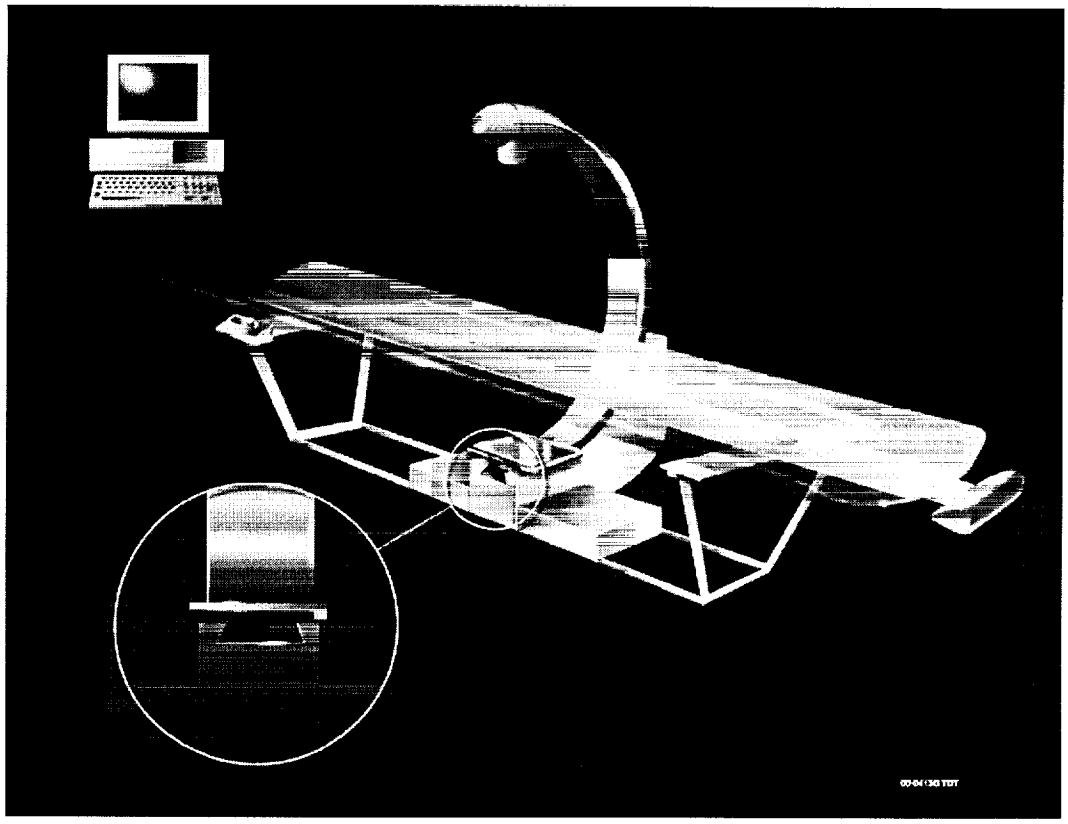


Figure 1. Artist's concept of space-based protoflight AMPDXA.

(6) *Revised program milestones*: In the initial proposal, we were focused on developing a form, fit function protoflight version of the AMPDXA. While this is still our ultimate goal, we recognize that several issues have to be resolved before an effective protoflight AMPDXA can be developed. These include: (1) demonstration that multiple projection technology improves BMD accuracy and collects structural details, (2) the structural details can be converted into bone

reconstruction models that preserve mechanical behavior, (3) soft tissue can be distinguished from bone and decomposed into fat and muscle, and (4) data can be collected reliably and repeatably on human subjects. To answer these questions, both a Laboratory Test Bed and a Clinical Test System have been constructed and are operational. These units are described in detail in the Accomplishments section below.

C. ACCOMPLISHMENTS

C.1 Design Strategy

While measurements of bone and muscle mass are statistically correlated with mechanical strength, they are not direct measurements of strength nor can they be directly employed in any conventional engineering analysis. As currently configured, bone and muscle mass measurements (via conventional DXAs or ultrasound) involve volumetric averaging which obscures structural details. Clearly, the mechanical consequences of lost bone and muscle mass is exhibited in the structure. To directly assess the biomechanical consequences of weightlessness, some absolute determination of skeletal mechanical competence is needed to supplement the conventional mass measurements. Engineering properties of the bones can be derived from bone mass data using DXA techniques. The method is based on geometrical methods (cross-sectional areas, moments of inertia, and bending moments) derived from the bone mass image. The technique was fully described in our research proposal.

Volume, weight, thermal characteristics, and power utilization are at a premium on both the space station and future interplanetary vehicles. However, the clinical requirements of a DXA scanner, particularly the ability to scan the entire body, require dimensions mandated by the source-detector geometry required for an efficient design. Because measurements involve standards with respect to bone mass and body composition, it is important that measurements are compatible with existing DXA systems. The scanner system must be extensively tested on earth using humans and measurement standards before use in space. Since the device has considerable commercial potential, the design must be suitable for earth-based application as a clinical DXA scanner. To reconcile these diverse requirements, the following design philosophy has been employed. In general, the basic sub-elements and geometry of the system have been designed to meet the general requirements for an earth-bound unit, but with the selection of materials, power utilization, and weight suitable for use on space vehicles. The design configuration was finalized and tested (using phantoms and other test objects) and a debulking effort was undertaken to reduce the projected volume and weight in the final protoflight configuration as much as possible, as shown in Table 1. Since the large area detector utilized the x-ray tube's output more efficiently, we have revised our geometry to design a scanner that has a source to detector separation of approximately 1 m. It utilizes a relatively narrow scanning C-arm gantry that can be stowed flat against a bulkhead during launch and when not in use. Conceptually, the unit was designed to require a minimal amount of assembly or "unfolding" for use. The scanner employs a rigid C-arm gantry that rotates to acquire the multiple projection angles. Patient translation, when necessary, is accomplished by a movable table. The expected use configuration is shown in Figure 1. The translation mechanism and table will be removable when not in use.

Clearly, the AMPDXA has significant commercial potential in the diagnosis and treatment (countermeasures) of osteoporosis. Some differences between the space-based and commercial versions will exist, but efforts are being made to ensure that the differences have no impact on measurement characteristics. Our design will always take into account that the unit will have extensive use during the bed rest studies being conducted to help learn about low gravity effects. We also will consider the possibility that an inexpensive, lightweight AMPDXA scanner would be welcome as a transportable diagnostic tool for use in physician's offices, clinics, and nursing homes.

C.2 Key Design Features

The spatial resolution for the amorphous silicon detector is better than 3.8 line pairs/mm with a quantum efficiency better than that of commercial image intensifier tubes. Comparable existing systems deliver a resolution between 0.1 and 1 line pair/mm, and the resolution is asymmetric in the scan plane (better in one dimension that in the other). Such behavior is shown in the BMD images of Figure 2 for the NSBRI AMPDXA and a commercial DXA scanner. This asymmetry causes unpredictable effects on bone geometry measurements that are not easily correctable in software.

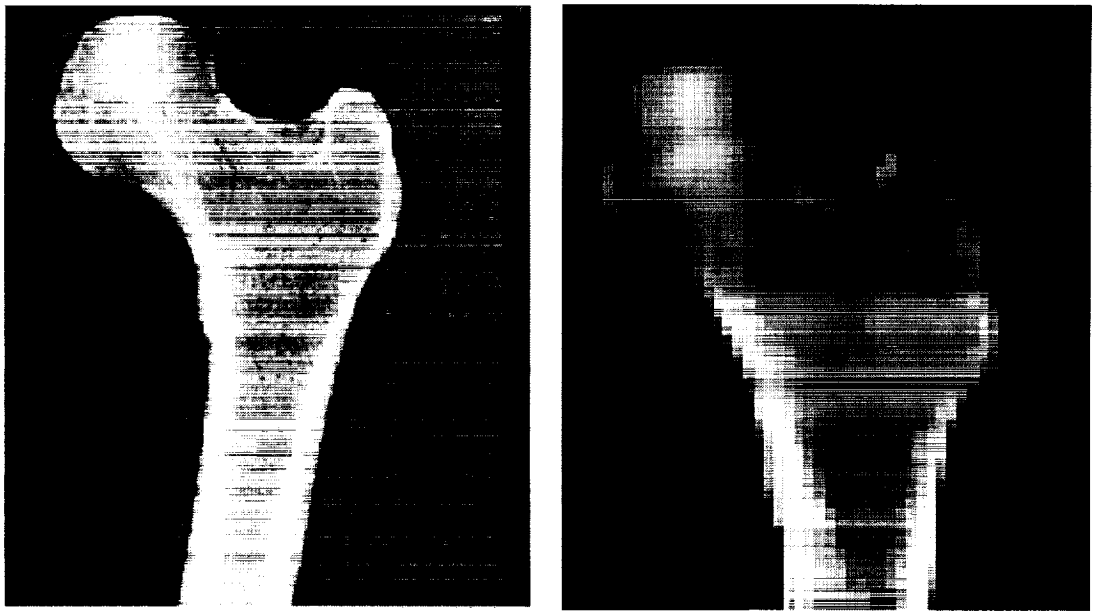


Figure 2. Comparison of (a) AMPDXA versus (b) conventional DXA (same bone).

X-ray production is a notoriously energy inefficient process, hence it is necessary to minimize the wastage of x-rays. Because of the larger detector and the need for fewer exposures per scan, the cone beam configuration of this design will be some 75 times more energy efficient than the fan beam system originally conceived. Power utilization will be significantly enhanced,

permitting the use of a lower power x-ray generator and affording some reduction in system mass.

The measurement uncertainty in a DXA scan is dependent on the x-ray energies used (determines "signal") and on the x-ray flux collected in each sample (determines "noise"). For a constant output x-ray system (most designs), the collected x-ray flux varies exponentially with patient thickness. Commercial DXA scanners use more than one scan speed to accommodate patient sizes, but usually the same scan speed is used with all patients. This means that measurement uncertainty is much worse on thick patients than with thin ones. The NSBRI AMPDXA scanner achieves a measurement uncertainty relatively independent of patient size. This is accomplished by modulating the x-ray flux to produce a constant average detector fluence, thus producing constant average noise level. Medical radiology and fluoroscopy systems use a similar technique called automated exposure control. With the use of digital image receptors, performing a low intensity "test" exposure prior to acquisition to gauge average patient transmission can do this. This is rapidly read out and the required exposure time computed. This can be done in less than 1 second and the data from the test exposure is summed to the actual acquisition so that there is no additional radiation required. An alternative scheme for exposure control based on a proprietary idea proposed by Varian is also being explored.

C.3 Radiation Safety

The major safety concern with the use of any x-ray system is the use of ionizing radiation, although DXA scanners, in general, require very small radiation doses. Subjects undergoing scanning will never the less be exposed to radiation, there exists some potential for exposure from scatter and leakage to those in close proximity during operation. A design goal is to provide effective doses to an average individual in the range of 5-10 mrads (0.05-0.1 mGy) or less for any scan sequence. The design limit for scatter and leakage will be less than 1 mrad (0.01 mGy) per minute of operation at a distance of 1 m from the gantry isocenter. Scan times for body subregions should be on the order of tens of seconds. To put the dose numbers in perspective, the average terrestrial dose-rate from natural background radiation is 300 mrad (3 mGy) per year in the U.S. The average dose-rate to Skylab astronauts was 71 mrad (0.71 mGy) per day, while that for the Apollo missions averaged 44 mrad (0.44 mGy) per day. All scanning modes should produce effective doses well under that received by astronauts in one day of spaceflight and, at most, during long flights, astronauts will be scanned no more often than biweekly or monthly. Our recent calculations and measurements indicate that with a single four-projection procedure consisting of a measurement at both the thigh and the calf (simultaneously using the large area detector), the total effective dose would be 0.33 mrem. Design issues influencing radiation doses to astronauts will be coordinated with radiation safety personnel at Johnson Space Center at critical junctures during scanner development.

C.4 Laboratory Test Bed

The Laboratory Test Bed (Figure 3), from which all the early images were derived (e.g., Figure 2(a)), is designed closely to match the geometry and operation of both the Clinical Test System and the envisioned protoflight unit. It utilizes Varian's VIP-9 detector with a cesium

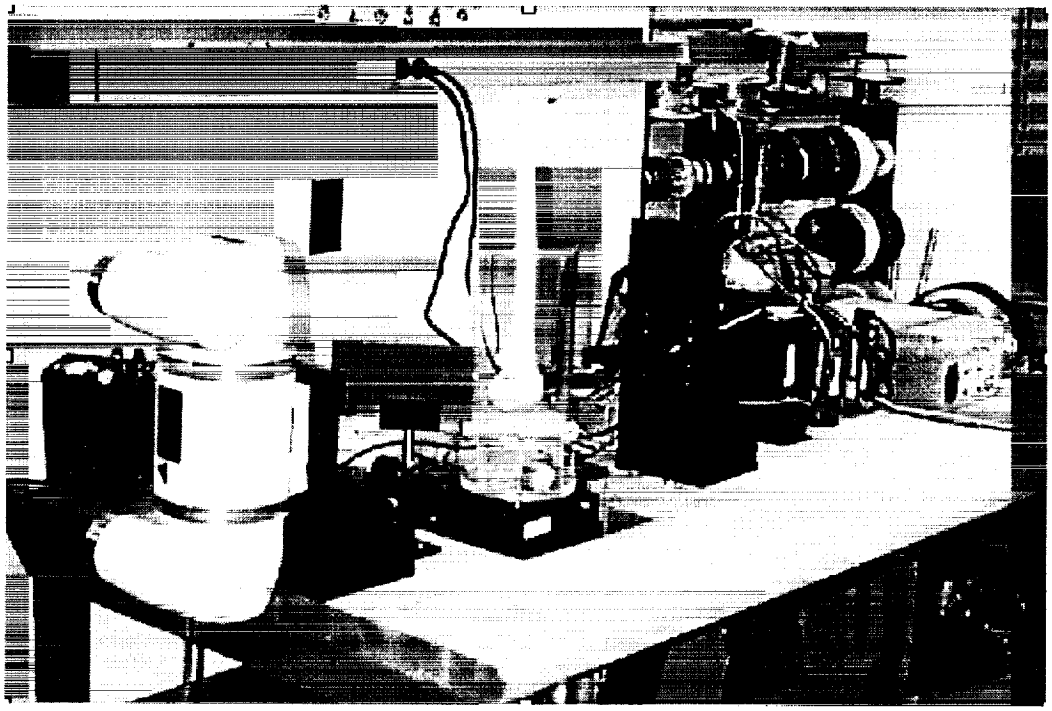


Figure 3. Laboratory Test Bed.

iodide scintillator, the same detector used in the Clinical Test System. The x-ray power supply (Model EDEC-80) and tube (Model A145) are commercial products supplied by Electromed International and Varian, respectively. Both the Laboratory Test Bed and the Clinical Test System utilize the same model power supply and tube. The protoflight system will use a lightweight tube (under development by Varian) and a custom-designed power supply described later in this article. As configured and filtered, the system produced x-rays at 80 and 140 kV_p. The important physical dimensions have been carefully matched; namely, the distance between the x-ray source and the isocenter and the source-to-detector distance. Instead of the gantry and table of the Clinical Test System, the Laboratory Test Bed has a precision turntable on a translating mount that allows the imaged object (everything from plastic bone models to human femurs and hams) to be positioned in the desired spatial orientation. The Laboratory Test Bed was used to perform the experiments necessary to develop the energy levels and filter combinations that were built into the Clinical Test System and will be used ultimately on the protoflight system, assuming no modifications are required after results from human testing are obtained. Since a conventional x-ray power source is being used, the versatility necessary to alter the energy levels and exposure parameters is retained. The Laboratory Test Bed can be used for pretesting and the results translated to both the Clinical Test System and the protoflight unit. In this way, the test parameters can be adjusted as needed without interrupting the clinical trials.

C.5 Clinical Test System

The Clinical Test System incorporates a high precision rotation and translation stage to provide the scanning capability to carry out qualification tests on human subjects. Because the Clinical Test System is to operate on earth, the table and gantry were not built to the mass specifications of the protoflight AMPDXA. In fact, the unit was built on a used CT scanner structure (Figures 4 and 5) which weights several hundred kilograms. Employing used equipment for some of the structural and rotating parts and machinery has allowed critical resources to be focused on the analysis and information extraction issues leading to clinical trials, as mentioned earlier. Since the initial focus of the development has been on bone, most of the preliminary data are on human bones and bone phantoms.

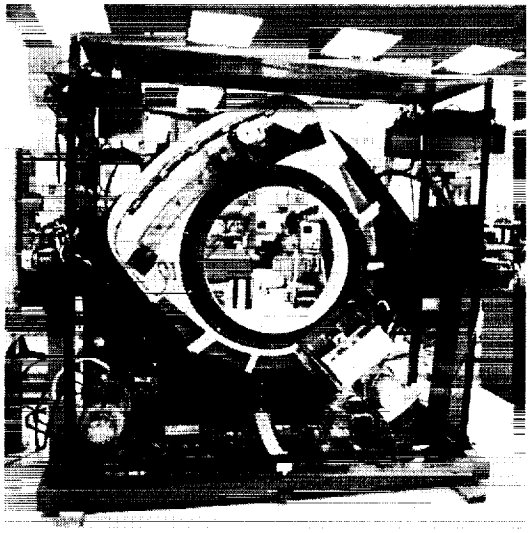


Figure 4. Clinical Test System gantry showing x-ray tube with collimator and filters and movable detector.

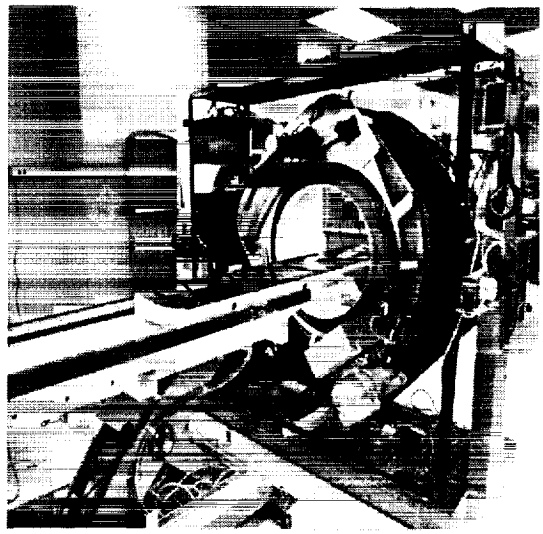


Figure 5. Clinical Test System with couch.

C.6 Imaging Results

The Laboratory Test Bed, in conjunction with the high-resolution detector and the bone analysis software that has been developed, has produced some exciting preliminary results. Figure 2(a) is a BMD image of a human femur immersed in a cylinder of water (water simulates the presence of muscle tissue). Figure 2(b) is the same bone shown imaged on a commercial DXA scanner (Hologic QDR 4500 at the Johns Hopkins Hospital) that is in regular service scanning patients and is typical of conventional, state-of-the-art commercial systems. The improvement in spatial and contrast resolution of the AMPDXA is quite evident by comparing the two images. This improvement is further illustrated by Figure 6, which shows bone mass distributions as a function of distance through a femoral slice in each of the two systems. Note the blurry image produced in the conventional DXA scanner (Figure 2(b)) generates a smeared mass profile. This effect is negligible in the AMPDXA image (Figure 2(a)) and, thus, it is not necessary to blur-correct the data to measure bone width. The high frequency content of the

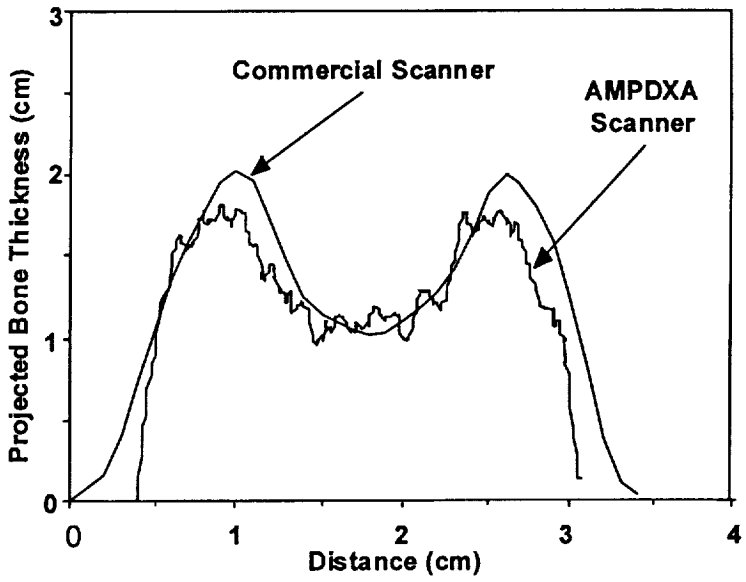


Figure 6. Bone mass profiles with distance across a given bone seciton.

AMPDXA profile is repeatable and provides additional information on the bone's microstructure.

Figure 7 provides a view of two such AMPDXA BMD profiles at different locations (cross sectional slices) on a femur. One aspect of dimensional measurements is that the image plane dimensions must be defined in the plane of the patient. Conventional DXA scanners, like the Hologic QDR4500, employ a fan beam that traverses down the patient to acquire the image in a series of pixel lines. Dimensions across the fan are known precisely as the line (y-pixel) spacing, but along the fan, the x-ray beam diverges so the geometric magnification of the scanned bone and thus x-pixel spacing varies depending on the bone position between the x-ray source and detector array. Hologic assumes a constant value for x-pixel spacing, but in practice this parameter varies with patient size. The AMPDXA scanner actually has this problem in both x and y directions in the image plane since the entire image is acquired in a single projection, using cone beam geometry. While it could be possible to assume a constant geometric magnification, we believe that this will be unsatisfactory for measuring subtle dimensional changes in bone that would be evident if countermeasures are working during long-term spaceflight. To solve the problem we will use the multiple projection capabilities of the AMPDXA to determine the magnification with a high degree of accuracy. From the beginning, the multiple projection capability was actually intended to provide true three-dimensional bone geometry, but, in addition, it provides the capability of determining magnification. Work on multiple image analysis has progressed quite well as shown in Figure 8, which illustrates BMD images of the same bone collected in seven different orientations. Using multiple projections about the bone axis allows structural properties (e.g., bending strength) to be obtained independent of patient position. To do this, we use a limited number of projections to derive, not a three-dimensional image, but sufficient information to specify the structural geometry in three

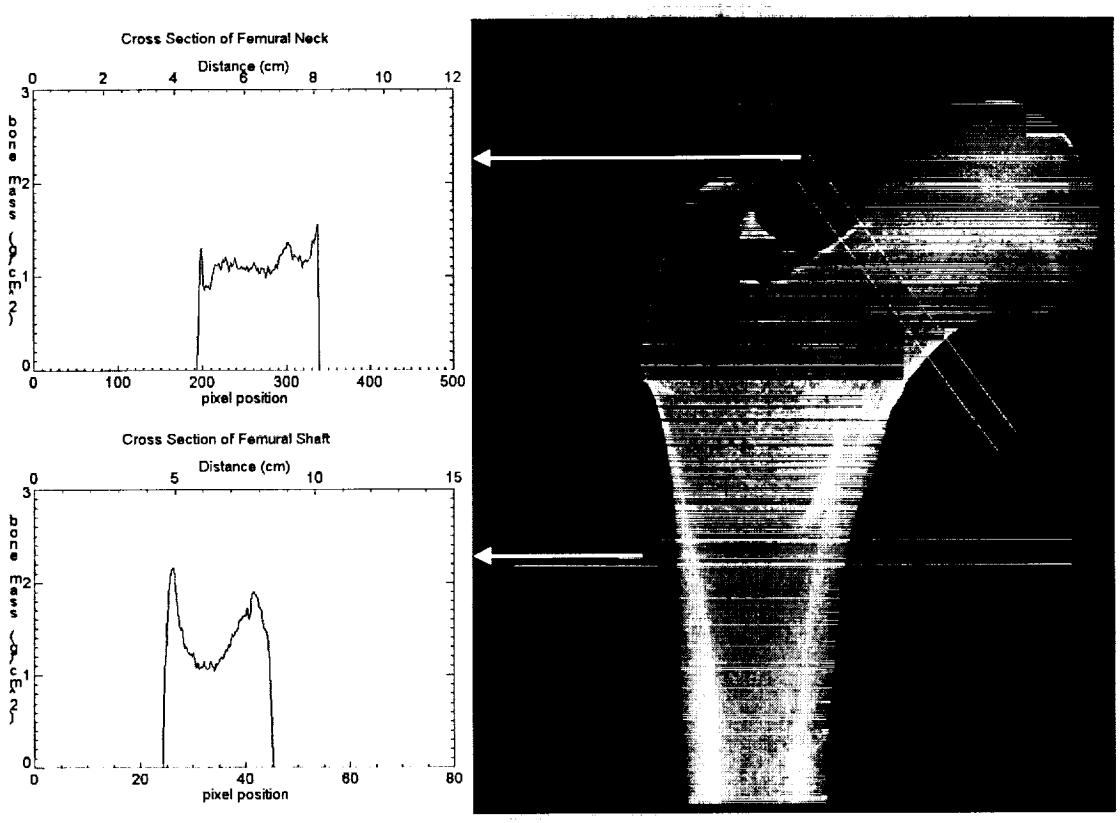


Figure 7. AMPDXA image showing location of analysis regions and the resultant mass profiles on the left.

dimensions. In principle, this can be done with as few as three projections over an arbitrary 90-degree rotational interval. This is based on the principles that:

1. Any two orthogonal cross-sectional moments of inertia (I_x and I_y) add to a constant (the polar moment of inertia).
2. A projection at a rotational angle between the two orthogonal projections can be used with the rotational axis principle to generate the product of inertia.
3. This can then generate the principle moments of inertia as:

$$I_{max} = \frac{I_x + I_y}{2} + \sqrt{\left(\frac{I_x - I_y}{2}\right)^2 + I_{xy}^2}$$

$$I_{min} = \frac{I_x + I_y}{2} - \sqrt{\left(\frac{I_x - I_y}{2}\right)^2 + I_{xy}^2}$$

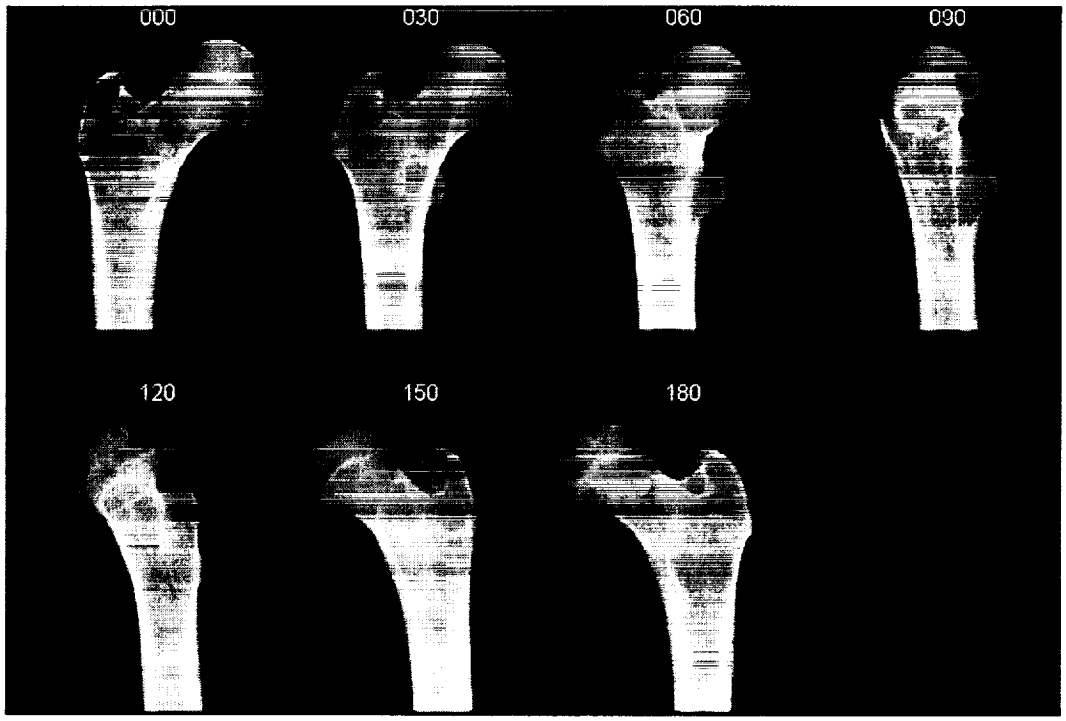


Figure 8. Multiple DXA projections using rotational stage.

An experiment was conducted with a cadaver femur using sets of 3 projections over 90 degrees, with an intermediate projection at 30 degrees relative to the first. Four sets of projections were obtained at 5 mm intervals from the base of the femoral shaft moving upward. The average I_{max} and I_{min} values are plotted with standard deviations in Figure 9.

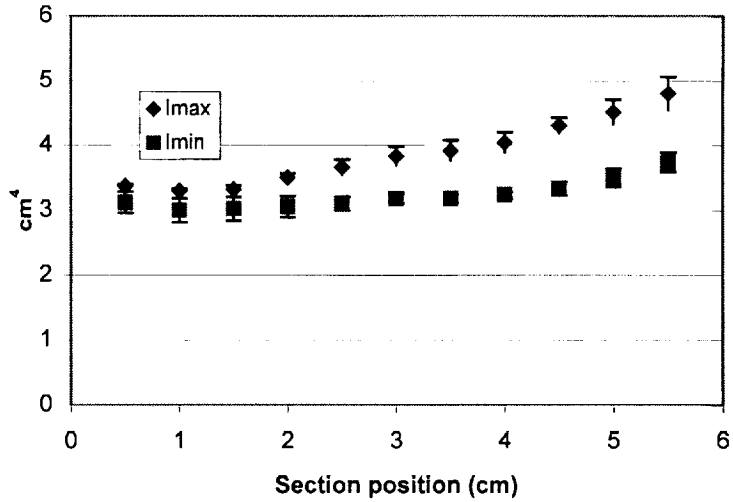


Figure 9. Principal moments of inertia at 5 mm intervals along the femoral shaft. Means and standard deviations are plotted for four sets of three projections.

The average coefficient of variation in I_{max} and I_{min} were 2.9% and 3.9%, respectively. Additional projections (above three) reduce this number further. Our experimental systems also have some known non-linearities which, when removed, will drop the error in the three projection estimation of moments to less than 1%.

The major advantage of this approach is that it can define the bending and torsional properties of a bone for loading in any conceivable configuration. The use of three projections may not be adequate in cases where there are two bones such as the forearm or lower leg because the two bones overlap in certain projections. We have done simulations that show that five projections are adequate in those cases, though we have not yet tested the algorithm with the AMPDXA.

The main disadvantage of the technique for deriving principle moments of inertia is that it can only be used in locations where projections over 90 degrees can be obtained with rotation (approximately) about the long axis of the bone. Furthermore these projections must be obtainable without superimposing other bones over regions of interest. Currently we have not been able to make the algorithm work in the femoral neck which extends medially at a ~45° to the long axis of the body and the scanner rotational axis. It may ultimately be possible to solve this problem with a complex rotation but in the interim, we are trying an alternative approach that shows some theoretical promise.

Since we only want to know whether the bone at the scanned site is structurally weak and whether there have been significant changes in strength since the previous measurement, an absolute image plane approach might be possible. If we define an absolute image plane in a subject such that the comparable plane can be imaged in all subjects then measurements in that plane could be compared between individuals. Furthermore if that plane could be reproducibly imaged, then any dimensional changes detected would be due to bone response and not measurement error. The ideal plane for imaging the hip is the plane of the neck-shaft angle. We believe that this plane can be defined in space by the use of two projections at different rotational angles. These two projections would be used to determine the three-dimensional location of the linear axis of the femoral shaft as well as the location of the spherical center of the femoral head. The point (femoral head center) and line (shaft axis) would define in space the location of the image plane. The scanner would then be rotated to a position perpendicular to this plane where the DXA image would be acquired. We have done some theoretical simulations of this method and have begun writing the algorithm. Testing will be done initially with the cadaver femur with the bench-top scanner, but the real test of the method will have to be done in live human subjects.

When a complex, three-dimensional bone is projected into a plane, the projected dimensions can change, depending on the projection direction. This is known as the two-dimension limitation. This is particularly problematic in the hip, the gold standard location for bone mineral analysis. DXA images are intended to be made with the projection perpendicular to the plane defined by the axes of the neck and shaft. Anatomically this plane is rotated back (anteverted) from the frontal plane of the body, about a center of rotation in the femoral head. The degree of anteversion varies between individuals, ranging from 10 to 30 degrees. In normal DXA scanning the patient's femur is rotated by the scanner operator some nominal amount (~15

degrees) in an attempt to put the neck-shaft plane in the image plane. Variations in femur rotation in practical DXA scanning place a limit on the level of inter-scan precision and accuracy that can be achieved, particularly in the measurement of geometry from projected dimensions.

An obvious way to solve the two-dimension limitation would be to generate three-dimensional images, thus completely specifying the cross-sections. This is possible with the AMPDXA because it can rotate about the patient and provide a sufficient number of projections to reconstruct the volume using the principles of cone-beam computed tomography. We have shown that the three projections are not sufficient for total image reconstruction. It does appear that a cone-beam type reconstruction from as few as three to seven projections is sufficient to produce a pseudo-three-dimensional geometry that is mechanically equivalent to the measured hip. A seven-projection cone-beam reconstruction is shown in Figure 10.

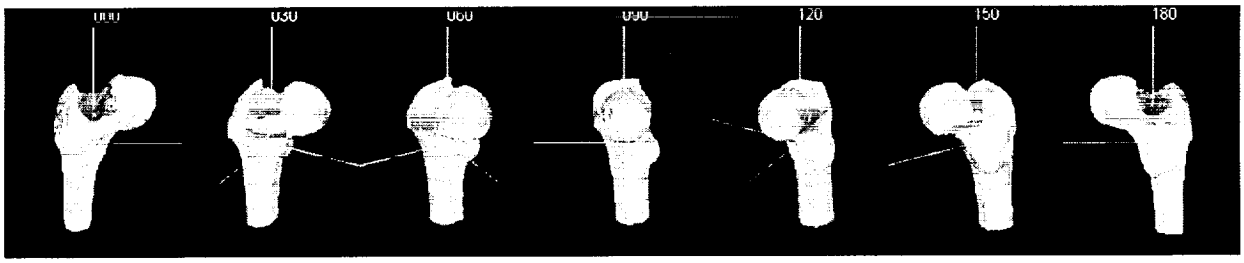


Figure 10. Cone-beam reconstruction from minimum projection data. Seven projections were utilized in this unfiltered reconstruction.

The results are reasonably good and could be improved further by adding more projections and by further modification of the reconstruction algorithm. However, for the specific purposes of imaging the geometry of the hip, true three-dimensional reconstructions may not be practical. In the body, the hip joint includes the massive pelvis. Any reconstruction of a femur could not exclude the pelvis and would necessarily also include the opposite femur in transverse projections. Since all materials in the reconstruction must be present in all projections, this would greatly increase the complexity of the problem. The present field dimensions accommodated by the AMDXA imaging panel will not encompass the entire pelvis or other regions without including use of a complex scanning motion for each projection. Secondly, the radiation dose penalty would be considerably higher due to the large number of projections required. Some CT capability may ultimately be desirable for some applications of the space-based version of the AMPDXA, but for the task of measuring hip geometry, may not be the optimal approach.

Densities obtained with the DXA are areal densities with the layering of bone, muscle, and fat confounding the data reduction and estimate process. Typically, the soft tissue on the top and bottom of the bone is assumed to have the same composition as on the sides. One fundamental difficulty with DXA is tht it is necessary to resolve three components (bone mineral contant (BMC), lean tissue mass (LTM), and fat tissue mass (FTM)) from only two measurements taken in a single plane. Consequently, additional assumptions must be made, typically reduction of data assumes that if the attenuation at a particular pixel is above a given

threshold, the tissue consists of bone and, possibly, soft tissue. An implicit assumption that must be made is that the attenuation of the soft tissue component in that pixel is equivalent to that in the tissue surrounding the bone. Nonetheless, DXA can be used to meaure or derive the following parameters: percent fat of soft tissue and soft tissue plus bone, soft tissue mass (FTM plus LTM), FTM, LTM, BMC, and total mass (soft tissue mass plus BMC). An example of a preliminary decomposition of an AMPDXA ham image into soft tissue mass and bone mineral density is sown in Figures 11, 12 and 13.

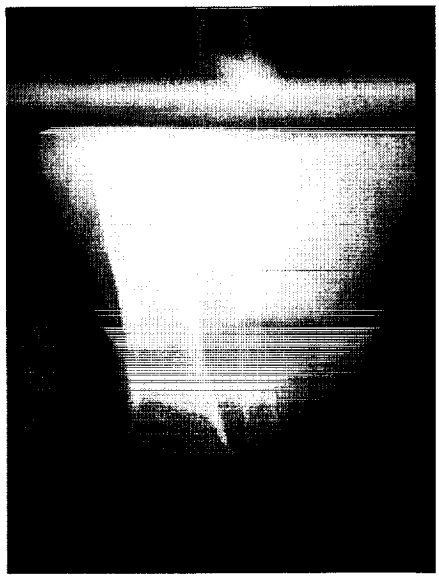


Figure 11. AMPDXA radiograph of a ham.

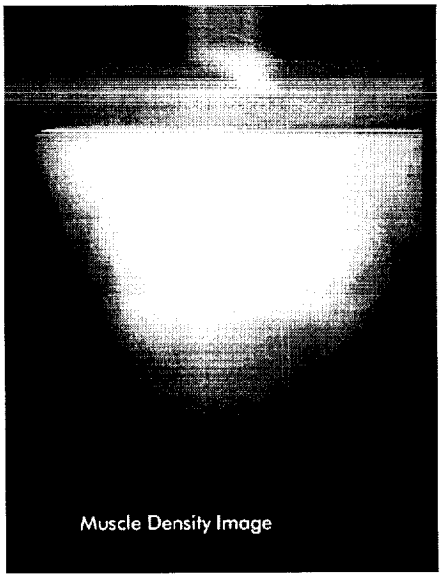


Figure 12. AMPDXA muscle tissue density image of a ham.

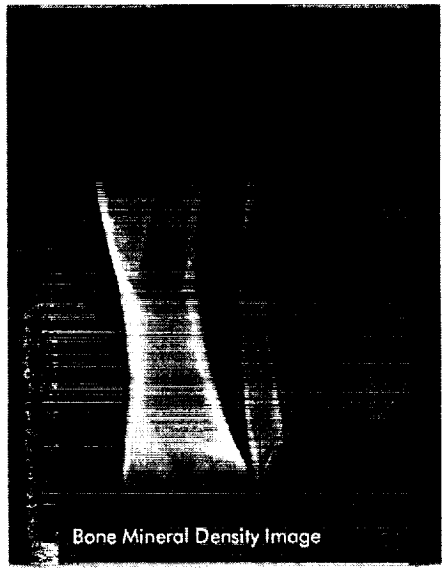


Figure 13. AMPDXA bone mineral density image of a ham.

C.7 Calibration and Optimization

The algorithms implemented to date for the AMPDXA are based on the original bone mass measurement techniques. These work reasonably well for bone but are not sufficiently accurate for soft tissue discrimination. To discriminate soft tissue we will employ the basis-material decomposition method originally described by Alvarez and Macovski [7]. This method assumes that x-ray images acquired at two energies can be decomposed into equivalent images consisting of thickness of two known basis materials e.g., aluminum and methyl methacrylate (acrylic). Calibration involves imaging of calibration phantoms consisting of a set of orthogonal thicknesses of aluminum, spanning the range of equivalent attenuation observed under clinical conditions. The calibration phantom used is one made in our laboratory. It is modeled after commercially available units and consists of 11 thicknesses of acrylic in 2.54 cm steps from 0 to 25.4 cm and 7 thicknesses of aluminum from 0 to 3 cm, for a total of 77 combinations. Our calibration phantom is shown in Figure 14. Our early efforts are based on the fitting of the data of the form:

$$H_l = k_1 A_1 + k_2 A_2 + k_3 A_1^2 + k_4 A_2^2 + k_5 A_1 A_2$$

$$H_h = k_6 A_1 + k_7 A_2 + k_8 A_1^2 + k_9 A_2^2 + k_{10} A_1 A_2$$

where A_1 and A_2 are the respective thickness of aluminum and plastic, respectively, and H_l and H_h are the negative natural logarithms of transmission (attenuation) at the low and high x-ray energies, respectively. These polynomials are then inverted to solve for the thicknesses as a function of the attenuation values as:

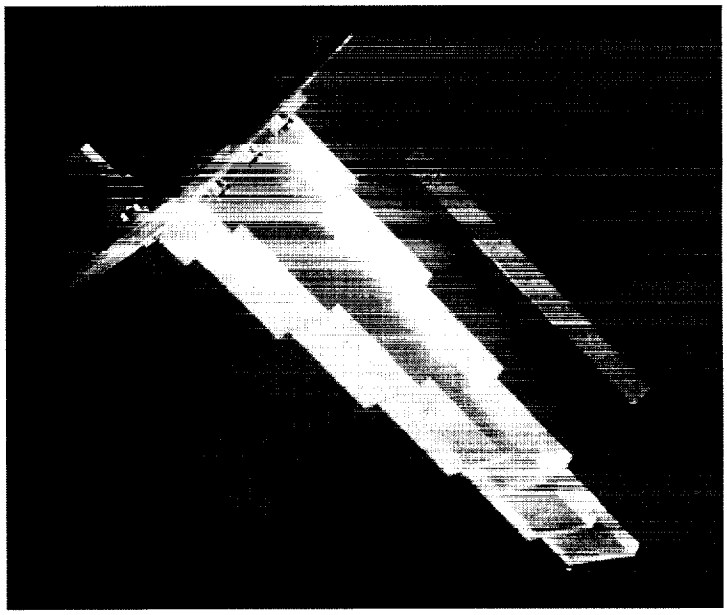


Figure 14. Calibration phantom.

$$A_1 = q_1 H_l + q_2 H_h + q_3 H_l^2 + q_4 H_h^2 + q_5 H_l H_h$$

$$A_2 = q_6 H_l + q_7 H_h + q_8 H_l^2 + q_9 H_h^2 + q_{10} H_l H_h$$

This is not a completely satisfactory method and is somewhat subject to systematic errors. We are in the process of modifying the algorithm using the analytical approach employing conic surface equations as proposed by Cardinal and Fenster [8]. This latter method has not yet been implemented; moreover the soft tissue calibration has not yet been completely optimized. An additional issue is that there is a non-linearity present in the AMPDXA system due to incomplete scatter removal. The first calibration images were made with a single, aluminum interspaced 10:1 grid with a 15 cm air gap. The scatter in the exit beam had large negative effects on the image data. Scatter effects can be minimized with knowledge of the scatter point-spread function, but this is difficult to implement in practice. The preferred method is to remove scatter before it is recorded. We have done some imaging utilizing a pair of better quality anti-scatter grids in a crosshatch configuration and found much improved linearity of the data. However, it is still possible to further improve the precision. This can be achieved by using either of two advanced, two-dimensional, anti-scatter grids with performance superior to conventional grids. These have been designed and are being constructed at the APL using advanced fabrication techniques. Theoretical simulations indicate that these grids will be able to produce higher contrast levels with a lower patient dose penalty compared to conventional grids. An example of a three-dimensional grid produced by electrical discharge machining and chemical etch of lead is shown in Figure 15.

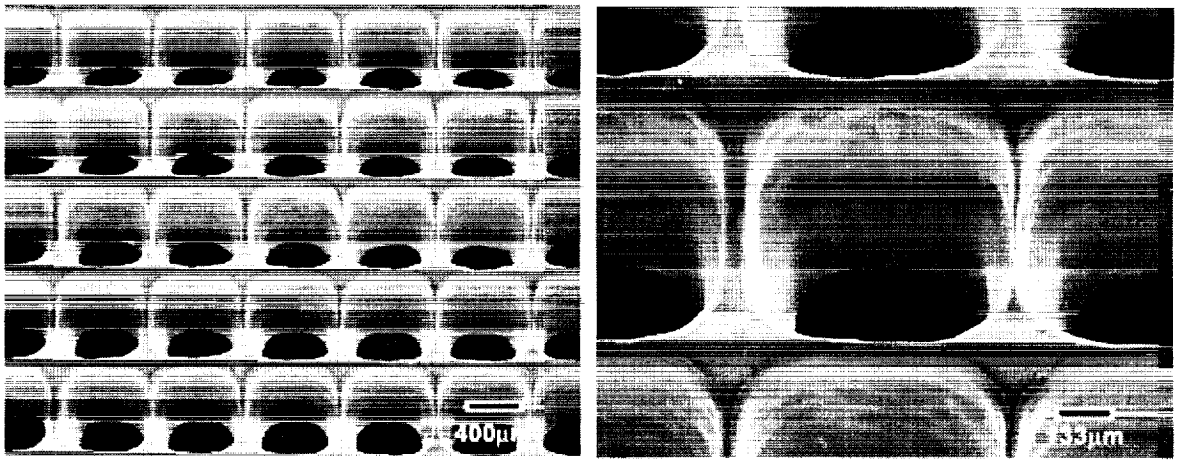


Figure 15. Examples of three-dimensional grid produced by electrical discharge machining and chemical etch of lead.

The calibration and optimization of the system will require a series of careful characterization and validation experiments prior to use on animal parts and human subjects. This effort will involve a full characterization of the noise and signal properties of the system as it is currently configured. Validation testing will involve known calibration materials simulating muscle, fat and bone with different thicknesses simulating the range of anticipated clinical

conditions. Bone mass will be simulated by samples of calcium phosphate tribasic type IV, lean tissue mass will be simulated by samples of 0.6% sodium chloride solution, and fat tissue mass will be simulated by samples of stearic acid.

C.8 Optimizing the High Voltage Power Supply for Space

To attain the high voltage and high power necessary to generate the x-rays and have a lightweight power supply, the design must incorporate circuitry not usually found in diagnostic x-ray systems. Instead, techniques used in avionics and lightweight radar transmitters will be employed.

There are three modes of operation required. First, since rotating anodes tend to crack if asked to absorb high-energy exposures while at low temperatures, warm up exposures must be made. In the typical diagnostic x-ray system these exposures are 80 kVp, 150-200 mA, and 1 second duration. Two of these 12-16 kW exposures are made using the large focal spot in order to bring the anode gently to a uniform, elevated temperature. This process is simple in ground based systems where peak powers of 80-100 kW are routine, but very costly in size and weight for a space-based system. Instead, we propose to have a second filament in the x-ray tube that will result in a very large focal spot and can be activated at much lower power levels to, albeit slowly, warm up the anode. This process will require only 3 mA at 80-90 kVp, or less than 300 watts, for less than one minute. A supply of this capacity and voltage should weigh less than 4 pounds. It will be switched into the circuit when required and then turned off and switched out by high voltage contactors when the tube is properly prepared for diagnostic exposures.

The pulsatile requirements from the power supply are very complex. Based on measurements made on the Laboratory Test Bed, we believe that typical exposures will be 80 kVp, 7.5 mAs (600 Joules) for the low energy and 140 kVp, 4 mAs (560 J) for the high energy condition. The typical diagnostic x-ray power supply (called the "generator" in diagnostic, medical x-ray parlance) would operate at peak powers of 5-20 kW and draw all of this power directly from the AC mains. Further complications arise in the power supply design when the need for variable exposure times and/or peak currents is factored in. This need is created by the requirement that each exposure drive the detector exactly to its limit to maximize the dynamic range in spite of the differing average radiopacity of different body parts and different patients. The power system must be capable of having either a way of stopping the exposure when the limit is reached (called automatic exposure control) or presetting the exposure magnitude by computer control. The generator used in the Clinical Test System can meet all of the above needs but weighs over 1500 pounds. Clearly, neither the "normal" x-ray generator nor the normal method of controlling the x-ray tube will be suitable for the advanced, space-based system.

Our proposed system will break up the x-ray tube's high voltage pulse into many short pulses of constant magnitude (depending on which energy level is selected). Choosing the number of short pulses integrated onto the detector will create the selectable exposure levels. After the first one or two short pulses, the detector will be read out and the peak pixel value within the patient image will be read out. The system will then compute the number of pulses

required and trigger the power supply the appropriate number of times. Certainly much calculation and experimentation needs to be done, but current thinking is that the short exposure should be on the order of 50 to 100 microseconds per pulse. The exposure length will be determined by a pulse forming network (PFN), a system of capacitors and inductors that will store the energy and shape its delivery into the square pulse. The energy will be stored at a relatively low voltage, perhaps 10 kV to minimize spacing requirements so that size and weight are minimized. A pulse transformer will then step up the output of the PFN to the required x-ray tube voltage. Only the top of the pulse transformer and the anode of the x-ray tube will be driven to the full 140 kV potential. There are many tradeoffs yet to be studied. For example, the pulse transformer ratio can not be made arbitrarily high without negatively affecting pulse shape. This clearly affects the output level of the direct current supply that charges the PFN. The output level will have an effect on size and weight. All must be studied and reasonable compromises will be made.

We expect that the astronauts imaged in the AMPDXA will be cooperative and relatively healthy so we expect little patient motion. The experience of current ground-based DXA units is that even those patients, not all of whom are in good health, are not very likely to be moving during the exposure (current DXA scanners use exposures in the tens of seconds). Therefore, we can operate the system at relatively low peak power levels and expand the exposure time in order to get adequate signal to noise ratios. We believe that setting the x-ray tube current to approximately 25 mA for the 80 kVp exposures would typically require 3000 exposures of 100 μsec each. In order to keep the peak power requirements of the DC power supply within reasonable bounds, we would allow a 1 millisecond interval between each of the short pulses. Therefore, the 80 kVp exposure would take 3 seconds, but the output of the DC power supply during the exposure would only be 200 watts. A power supply of this capacity would likely weigh less than 10 pounds. Several pounds additional are required for the pulse transformer and the filament transformer and power supply. The pulse forming network will add less than one pound.

In order to operate properly during the 140 kVp exposures, several additional adjustments must be made. Instead of changing the output of the DC power supply which would make regulation of its output more difficult, the PFN would drive a different tap on the pulse transformer so its ratio would be, for example, 14:1 instead of 8:1. The x-ray tube current must be changed to maintain the same impedance reflected at the PFN as it was during 80 kVp operation. Thus, the tube current will be 14.3 mA during 140 kVp operation. The pulse length will remain at 100 μsec and we estimate that 2800 exposures would typically be required, taking 2.8 seconds. The power required from the DC power supply during 140 kVp operation will be the same as during 80 kVp operation. This process would be repeated for each projection with several seconds between projections necessary for rotating the imaging system.

II. IMPLICATIONS FOR FUTURE RESEARCH

The AMPDXA project has many implications for future research and development. The AMPDXA, as described above, has direct application to risk reduction in NASA's Critical Research Path. The AMPDXA is capable of real-time monitoring of bone and muscle loss at extremely high precision. Since the results are patient-specific and not tied to volumetric averages and statistical norms, the AMPDXA is a very useful tool for monitoring the effectiveness of countermeasures as well as determining risk of fracture under various loading conditions and activity scenarios. The AMPDXA also appears to be a natural adjunct to earth-bound research on the effect of aging and disuse on bone integrity. It could also be used as a routine screening tool for osteoporosis and as a monitoring instrument for osteoporosis drug therapy.

A mentioned above, the availability of an advanced, multiple projection DXA scanner with high precision has significant potential for fostering future musculoskeletal research in a number of disciplines. The significant improvements in scanning precision and accuracy offered by the AMPDXA could have major impact on the clinicians' ability to reliably quantify subtle changes in bone mass at any anatomical location. With current commercial DXAs, observations are made over periods of a year or more in order to ascertain the efficacy of a given treatment, i.e., whether a patient's bone mass is increasing or decreasing. The AMPDXA's higher measurement precision can reduce the observational period for follow-up, significantly improving the accuracy and efficacy of treatment.

Furthermore, the ability to define the structural geometry of bones would provide the clinician with heretofore unavailable information to determine the biomechanical implications of a given change in bone mass. Current scanners do not provide information leading to a direct measurement of bone strength (bone mass or density are not strength properties). The NSBRI AMPDXA system is designed for combined measurements of bone and muscle geometry. There is a growing realization that muscle forces and alterations in muscle function are important determinants of skeletal integrity in both disuse osteoporosis as well as for the osteoporosis of aging.

The ready availability of the highly accurate AMPDXA will significantly aid in the development of countermeasures. The real-time feedback available to the astronauts and ground-based doctors from the spaceflight version would be a significant help to today's clinician trying to assess the efficacy of a particular countermeasure. Because of the accurate quantitative measurements made by the AMPDXA, even small improvements in remediation could be observed.

To bring the AMPDXA to its full potential, the following specific research problems must be addressed:

1. Human testing with the Clinical Test System to develop the final measurement parameters (e.g., dose, duration of x-ray pulses, final filters, operational scenarios, etc.).

2. Refine software algorithms for the extraction of soft tissue.

3. Solving the two-dimensional versus three-dimensional reconstruction problem.

4. Refine software algorithms for relating bone strength to risk of fracture.

5. Build and test the lightweight power supply.

6. Software (and instrumentation) refinements to allow the collection of radiographs for diagnosis of injury and disease.

Commercially, if cost projections could be realized, there is a significant market for population screening and treatment monitoring of osteoporosis in postmenopausal women. It is believed that a compact, easy to use, vertical instrument (patient stands) could be developed for the small clinic or doctor practice. The market for this is several hundred to several thousands of units, depending on final cost. We intend to continue our discussions with Norland and Hologic regarding the commercial potentials. Structuring the scanner as a ground-based, transportable unit has large implications for use as a tool in nursing homes, clinics, and medical offices. Dr. Adrian LeBlanc and Dr. Jay Shapiro have already indicated that a precision AMPDXA would greatly assist their work in bed rest bone loss studies.

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APPENDIX A

Project Research Data

Research data as appropriate has been incorporated into the main report.

APPENDIX B

Listing of Publications, Presentations, and Inventions

Publications and Presentations

- T. J. Beck, H. K. Charles, Jr., H. S. Feldmesser, V. L. Pisacane, "Multiple Projection DEXA Scanner for Measuring Structural Geometry," *American Society of Bone and Mineral Research Meeting*, San Francisco, December 1998.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, and V. L. Pisacane, "Compact, High Precision, Multiple Projection DEXA Scanner for Measurement of Bone and Muscle Loss During Prolonged Spaceflight," *Proc. First Biennial Space Biomedical Investigator's Workshop*, League City, TX, January 11-13, 1999, pp. 202-204.

- Posters at: NASA-JSC's Inspection '98 (October 1998)
  1998 Maryland Technology Showcase (8-9 December 1998)

- *APL News* Article, Feb 1999, Vol. 55, No. 1-2.

- National Public Radio Interview part of program on Space and Aging "Soundprint," February 27, 1999.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, T. S. Spisz, and V. L. Pisacane, "Multiple Projection DEXA Scanner for Precision Bone and Muscle Loss Measurements and Analysis During Prolonged Spaceflight," *Proc. Space Technology and Applications International Forum (STAIF-2000)*, M. S. El-Genk, Editor, Albuquerque, NM, January 30-February 3, 2000, pp. 300-305.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, T. S. Spisz, and V. L. Pisacane, "Precision Bone and Muscle Loss Measurements by Advanced Multiple Projection DEXA (AMPDEXA) Techniques for Spaceflight Applications," Proc. 13th IAA Humans in Space Symposium, Santorini, Greece, 20-26 May 2000, 3 pages.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, T. S. Spisz, and V. L. Pisacane, "Advanced Multiple Projection DEXA (AMPDEXA) Scanner for Precision Bone and Muscle Loss Measurements," *Proc. 22nd Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, Chicago, IL, 23-38 July 2000, (abstract only).

Inventions

- "Light Weight, Compact, High-Precision, Multiple Projection DEXA Scanner System," Harry K. Charles, Jr., Thomas B. Beck, Howard S. Feldmesser, and Thomas C. Magee, December 14, 1998.

- "Cross-Hatched, Focused, X-ray Scatter Reduction Grid Fabrication by Lamination Techniques," H. S. Feldmesser, P. R. Falk, T. J. Beck, and H. K. Charles, Jr., December 1999.

- "Three Dimensional X-ray Antiscatter Grid Using Micromachined and Chemically Polished Lead Sheets," H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, D. M. Lee, D. J. Dawson, J. A. Weiner, and C. E. Bennett, August 2000.

APPENDIX C

Copy of Publications

- T. J. Beck, H. K. Charles, Jr., H. S. Feldmesser, V. L. Pisacane, "Multiple Projection DEXA Scanner for Measuring Structural Geometry," *American Society of Bone and Mineral Research Meeting*, San Francisco, December 1998.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, and V. L. Pisacane, "Compact, High Precision, Multiple Projection DEXA Scanner for Measurement of Bone and Muscle Loss During Prolonged Spaceflight," *Proc. First Biennial Space Biomedical Investigator's Workshop*, League City, TX, January 11-13, 1999, pp. 202-204.

- *APL News* Article, Feb 1999, Vol. 55, No. 1-2.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, T. S. Spisz, and V. L. Pisacane, "Multiple Projection DEXA Scanner for Precision Bone and Muscle Loss Measurements and Analysis During Prolonged Spaceflight," *Proc. Space Technology and Applications International Forum (STAIF-2000)*, M. S. El-Genk, Editor, Albuquerque, NM, January 30-February 3, 2000, pp. 300-305.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, T. S. Spisz, and V. L. Pisacane, "Precision Bone and Muscle Loss Measurements by Advanced Multiple Projection DEXA (AMPDEXA) Techniques for Spaceflight Applications," Proc. 13th IAA Humans in Space Symposium, Santorini, Greece, 20-26 May 2000, 3 pages.

- H. K. Charles, Jr., T. J. Beck, H. S. Feldmesser, T. C. Magee, T. S. Spisz, and V. L. Pisacane, "Advanced Multiple Projection DEXA (AMPDEXA) Scanner for Precision Bone and Muscle Loss Measurements," *Proc. 22nd Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, Chicago, IL, 23-38 July 2000, (abstract only).

Multiple Projection DEXA Scanner For Measuring Structural Geometry

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Abstract

Work in our laboratory using commercial DEXA scanners has shown that bone strength defining details underlying observed changes in bone mass due to age, disuse, and microgravity are evident in the structural geometry, i.e., the quantification of bone size and shape. Commercial DEXA scanners can be used to measure geometry from mass data and image dimensions, but are optimized for measurement of bone mass, not dimensions. Precision and accuracy of current systems are limited by poor spatial resolution and constraints of two-dimensional projections of three-dimensional bones. A whole-body, fan-beam DEXA scanner was therefore designed specifically for bone geometry measurements. The scanner is based on a kV/filter switching X-ray generator and a high-resolution detector employing a high quantum efficiency scintillator coupled via a novel optic coupler to an array of charge-coupled devices (CCDs). The design incorporates rotational capability to provide multiple projections so that complex, cross-sectional geometry can be evaluated in three dimensions. Algorithms for the derivation of three-dimensional geometry from as few as three projections have been implemented. A theoretical analysis of performance indicates that bone mass precision will exceed that of commercial DEXA systems and spatial resolution should be better than 3 line pairs/mm in the patient. Scanning attributes are incorporated to make measurement precision nearly independent of patient thickness.

A full-size, bench-top prototype of the scanner has been constructed for testing of principles and experimental verification of simulations. The prototype incorporates a high-precision rotation and translation stage to provide scanning capability. Full scale performance with a field width of 35 cm will require completion of the multi-module detector array, but assembly of a single CCD module has been completed and testing has begun. The single CCD will permit optimization of signal performance under simulated patient conditions as well as experimental verification of theoretical performance characteristics. System performance data will be presented and compared to theoretical predictions and commercial scanner performance levels. Images of human cadaver hip and spine specimens, together with mass and geometric measurements under simulated in vivo conditions, will also be presented.

American Society for Bone
and Mineral Research Meeting

December 1998
San Francisco, CA

COMPACT, HIGH PRECISION, MULTIPLE PROJECTION DEXA SCANNER FOR MEASUREMENT OF BONE AND MUSCLE LOSS DURING PROLONGED SPACEFLIGHT

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INTRODUCTION

The purpose of the Dual Energy X-ray Absorptiometry (DEXA) project is to design, build, and test an advanced X-ray absorptiometry scanner capable of being used to monitor the deleterious effects of weightlessness on the human musculoskeletal system during prolonged spaceflight. The instrument is based on the principles of dual energy X-ray absorptiometry and is designed, not only to measure bone mineral density and volume and to decompose soft tissue into measurements of fat and lean mass but also, to use those measurements to derive structural properties (cross sections, moments of inertia) permitting an assessment of the biomechanical upon consequences of microgravity on bone and/or muscle mass and the potential risk upon returning to planetary gravity levels. Multiple projection technology, coupled with axial translation, will be employed to provide geometric properties in three dimensions suitable for a three-dimensional structural analysis of the scanned region. The structural analysis will then be combined with bone models and projected loading scenarios to determine risk of fracture. The instrument will employ advanced fabrication techniques to minimize volume and mass (100 kg current target with a long-term goal of 60 kg) of the scanner as appropriate for the space environment, while maintaining the required mechanical stability for high-precision measurement. The unit will have the precision required to detect changes in bone mass and geometry as small as 1% and changes in muscle mass as small as 5%.

RESEARCH STATUS

From the earliest experience with prolonged weightlessness, it has become known that the elimination of gravitational effects on the human body produces an adaptation response resulting in the wasting of body skeletal muscle and bone mass. These musculoskeletal effects are somewhat complex because the stimuli for maintaining homeostasis of muscle and bone appear to be different; moreover, there also appear to be differences between losses in weight bearing and non-weight bearing body regions. Bone mass lost from the vertebrae, pelvis, and proximal femur average between and 1 and 1.6% per month. This loss magnitude should be contrasted to the loss from these sites of about 0.8 to 1.3% per year in postmenopausal women, and it is highly associated with increased bone fragility. The ultimate concern is that the loss of mass will lead to degradation in mechanical competence and possible failure. With restricted access to medical treatment during prolonged space travel, bone fracture may prove to be catastrophic, especially since healing rates in the absence of mechanical stimulus (load) are believed to be degraded. Clearly, effective countermeasures to stem the loss as well as some method for dynamically monitoring countermeasure effectiveness are required.

METHODS

While measurements of bone and muscle mass are statistically correlated with mechanical strength, they are not direct measurements of strength and cannot be directly employed in any

conventional engineering analysis. As currently configured, bone and muscle mass measurements (via conventional DEXAs or ultrasound) involve regional averaging which obscures structural details. Clearly, the mechanical consequences of lost bone and muscle mass are exhibited in the structure. To directly assess the biomechanical consequences of weightlessness, some absolute determination of skeletal mechanical competence is needed to supplement the conventional mass measurements. Engineering properties of the bones can be derived from bone mass data using DEXA techniques. The method is based on geometrical methods (cross-sectional areas, moments of inertia, and bending moments) derived from the bone mass image.

RESULTS

Work in our laboratory using commercial DEXA scanners has shown that bone strength defining details underlying observed changes in bone mass due to age, disuse, and microgravity are evident in the structural geometry; i.e., the quantification of bone size and shape. Commercial DEXA scanners can be used to measure geometry from mass data and image dimensions but are optimized for measurement of bone mass, not geometry. Poor spatial resolution and constraints of two-dimensional projections of three-dimensional bones limit precision and accuracy of current systems. A whole body fan-beam DEXA scanner was therefore designed specifically for bone geometry measurements. The scanner is based on a kV/filter switching X-ray generator and a high resolution, high quantum efficiency detector. Several different detector schemes are under consideration, including a conventional fan-beam scintillator-photodiode array, a high-quantum efficiency scintillator-coupled array of charge-coupled devices (CCDs), and a flat-panel detector based on amorphous silicon diode arrays.

The design incorporates rotational capability to provide multiple projections so that complex cross-sectional geometry can be evaluated in three dimensions. Algorithms for the derivation of three-dimensional geometry from as few as three projections have been implemented. A theoretical analysis of performance indicates that bone mass precision will exceed that of commercial DEXA systems, and spatial resolution should be better than three-line pairs/mm in the patient. Scanning attributes are incorporated to make measurement precision nearly independent of patient thickness.

A full-size bench-top test bed of the scanner has been constructed for testing of principles and experimental verification of simulations. The prototype incorporates a high-precision rotation and translation stage to provide scanning capability. Full-scale performance with a field width of 35 cm will require completion of the multi-module detector array, but assembly of a single CCD module has been completed and testing has begun. The single CCD will permit optimization of signal performance under simulated patient conditions, as well as experimental verification of theoretical performance characteristics. System performance data has been collected and is compared to both theoretical predictions and commercial scanner performance. Images of human cadaver hip and spine specimens together with mass and geometric measurements under simulated in vivo conditions will also be presented.

CONCLUSION

A compact, high precision, multiple projection DEXA scanner system is being built to accurately measure bone and muscle loss during prolonged spaceflight. The availability of such a system

offers significant potential for fostering future musculoskeletal research in a number of disciplines.

FUTURE PLANS

Evaluation of other detector designs will be completed during the next few months. Finalization of the high-voltage, power supply design will also be accomplished. Completion of these two items will allow detail design of the structure and control hardware and software to be developed. While final electronic and mechanical designs are being completed and construction of the hardware prototype is continuing, the test bed will be used to (1) further develop analysis algorithms, (2) develop the appropriate inputs for bone modeling software, and (3) validate the inputs and links to the risk of fracture information development activity.

The ready availability of a highly accurate DEXA will significantly aid in the development of countermeasures. The real-time feedback available to the astronauts and ground-based doctors from the spaceflight version would be a significant help to today's clinician trying to assess the efficacy of a particular countermeasure. Because of the accurate quantitative measurements made by our DEXA, even small improvements in remediation could be observed.

Since it is likely that people in long-term spaceflight might need additional diagnostic imaging capabilities, we will attempt to include additional features in the DEXA scanner. Conventional planar radiographic capability could be built into the scanner and would assist astronauts and earth-based medical personnel in diagnosing and monitoring fractures and other disease states that are imageable. Further work could be done to build in CT capability to extend this unit's utility.

Commercially, if cost projections could be realized, there is a significant market for population screening and treatment monitoring of osteoporosis in postmenopausal women. It is believed that a compact, easy-to-use, portable instrument could be developed for the small clinic or doctor practice. The market for this is several hundred to several thousand units, depending on final cost. We intend to continue our discussions with medical device and instrumentation firms regarding the commercial potential. Structuring the scanner as a ground-based, transportable unit has large implications for use as a tool in nursing homes, clinics, and medical offices.

Bone Structure Scanner Shows Strong Potential for Both Space and Commercial Use

Pamela Houghtaling (APL News, January 1999)

What do astronauts and an aging population have in common? Both groups face the potential for the loss of bone mass and, consequently, a risk of fracture, though for different reasons. One of the APL technology development projects funded by NASA's National Space Biomedical Research Institute (NSBRI) may well improve detection and monitoring of the problem through major changes in Dual Energy X-ray Absorptiometry (DEXA) scanner technology.

The new DEXA scanner is being developed as a compact, high-precision, multiple-projection instrument capable of providing images of bone structure not possible with today's technology. This R&D project has the potential to be turned into a qualified space instrument and commercialized for use as a diagnostic and screening tool for the general population.

Harry Charles of the Technical Services Department is serving as the principal investigator on the project and Thomas Beck of the Johns Hopkins School of Medicine, as the co-principal investigator. Charles describes himself as the builder of the instrument and Beck as the radiation physicist. Other members of the DEXA Team include Howard Feldmesser and Tom Magee of the Technical Services Department, and Vince Pisacane from the Directors' Office.

This DEXA scanner would enable medical personnel to look at what happens to bone and muscle structure during long-term spaceflight, such as a mission to Mars. Astronauts can lose from one to six percent of their bone mass a month in microgravity. The instrument could be carried onboard, enabling astronauts to take x-rays on a regular basis. This way, they could measure the risk of fracture and monitor the appropriateness and effectiveness of countermeasures.

Into the second year of a three-year NSBRI grant, Charles' team is working on the scientific and engineering issues of the new scanner. A test bed has been constructed. "Many questions must be answered before the instrument can be built," says Charles. "The goal is to make it five to ten times more accurate than what is commercially available today."

Current x-ray technology provides an average of the bone density in the scanned region, but not the structural details. The multiple-projection DEXA scanner will develop precise cross-sectional images in 3-D and, through engineering analysis, provide a clearer indication of bone strength and fracture risk.

In addition to questions concerning the level and source of the x-rays, design of this DEXA scanner presents structural and thermal challenges. In order to qualify for spaceflight, this instrument must be compact, lightweight, and stowable, and, at the same time, provide enough energy to generate X-rays at voltages exceeding 100,000 volts (peak). Space instruments typically are low-powered. In contrast, conventional x-ray machines are large, heavy, rigid structures and generate a lot of heat, which cannot be allowed to radiate inside the space cabin.

As Charles explains, the scanner calls for an innovative approach to the cooling of electronic equipment.

The new version of the DEXA scanner is expected to be commercially viable for use in medical offices, clinics, and nursing homes. Diagnosing and monitoring the treatment of osteoporosis will become more prevalent as the population ages.

The DEXA Team is discussing commercialization prospects with a number of medical device and instrumentation firms. APL and the School of Medicine have filed a joint patent application for the scanner.

National Public Radio (NPR) interviewed Charles last month for a documentary that will be aired in February.

Multiple Projection DEXA Scanner for Precision Bone and Muscle Loss Measurements and Analysis During Prolonged Spaceflight

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Abstract. Bone structural information derived from DEXA data is shown to be relevant in explaining BMD loss versus strength-related observations in both aging populations and individuals exposed to microgravity for prolonged periods. Commercial DEXA instruments are limited (and not optimized) to make these critical structural measurements. Progress on the development of a multiple projection DEXA scanner system for making precision bone and muscle loss measurements and their resultant implications on bone strength and fracture risk is described.

INTRODUCTION

The purpose of the Dual Energy X-ray Absorptiometry (DEXA) project is to design, build, and test an advanced X-ray absorptiometry scanner capable of being used to monitor the deleterious effects of weightlessness on the human musculoskeletal system during prolonged spaceflight. The instrument is based on the principles of dual energy X-ray absorptiometry and is designed, not only to measure bone mineral density and volume and to decompose soft tissue into measurements of fat and lean mass but also, to use those measurements to derive structural properties (cross sections, moments of inertia) permitting an assessment of the biomechanical upon consequences of microgravity on bone and/or muscle mass and the potential risk upon returning to planetary gravity levels. Multiple projection technology, coupled with axial translation, will be employed to provide geometric properties in three dimensions suitable for a three-dimensional structural analysis of the scanned region. The structural analysis will then be combined with bone models and projected loading scenarios to determine risk of fracture. The instrument will employ advanced fabrication techniques to minimize volume and mass (100 kg current target with a long-term goal of 60 kg) of the scanner as appropriate for the space environment, while maintaining the required mechanical stability for high-precision measurement. The unit will have the precision required to detect changes in bone mass and geometry as small as 1% and changes in muscle mass as small as 5%.

MEDICAL BACKGROUND

From the earliest experience with prolonged weightlessness, it has become known that the elimination of gravitational effects on the human body produces an adaptation response resulting in the wasting of body skeletal muscle and bone mass. These musculoskeletal effects are somewhat complex because the stimuli for maintaining homeostasis of muscle and bone appear to be different; moreover, there also appear to be differences between losses in weight bearing and non-weight bearing body regions (Oganov, 1992 and Vogel, 1976). Little bone mass appears to be lost from the upper extremities during weightlessness, while bone mass lost from the vertebrae, pelvis, and proximal femur average between and 1 and 1.6% per month. This loss magnitude should be contrasted to the loss

from these sites of about 0.8 to 1.3% per year in postmenopausal women (Wahner, 1996), and it is highly associated with increased bone fragility. The ultimate concern is that the loss of mass will lead to degradation in mechanical competence and possible failure. With restricted access to medical treatment during prolonged space travel, bone fracture may prove to be catastrophic, especially since healing rates in the absence of mechanical stimulus (load) are believed to be degraded (Kiratli, 1996). Clearly, effective countermeasures to stem the loss as well as some method for dynamically monitoring countermeasure effectiveness are required.

METHODS

It is a known fact that humans lose bone mass throughout their adult life and that within any given population age group there are a significant number of men and women with low bone mineral density (BMD); yet, most osteoporotic fractures occur at the end of life. Most researchers would attribute this phenomena to other factors such as propensity for falls, reduced bone quality, etc., but we strongly believe the explanation lies in the bone's structural details. Our methods are based on the hypothesis that throughout most of adult life, aging bones become more structurally efficient and retain their strength even though bone mineral density declines. The homeostatic mechanism for such strength maintenance depends on skeletal loading. Thus, to maintain bone strength, an accustomed level of loading on the skeletal system must be maintained. Absence of loading such as prolonged spaceflight (or disuse) can cause uncompensated loss of bone strength. Even reduced loading (caused by muscle wasting and inactivity in the elderly) can cause a disruption in the bone strength maintenance mechanism.

While measurements of bone and muscle mass are statistically correlated with mechanical strength, they are not direct measurements of strength and cannot be directly employed in any conventional engineering analysis. As currently configured, bone and muscle mass measurements (via conventional DEXAs or ultrasound) involve regional averaging which obscures structural details. Clearly, the mechanical consequences of lost bone and muscle mass are exhibited in the structure. To directly assess the biomechanical consequences of weightlessness, some absolute determination of skeletal mechanical competence is needed to supplement the conventional mass measurements. Engineering properties of the bones can be derived from bone mass data using DEXA techniques (Martin, 1984 and Beck, 1990). The method is based on geometrical measurements (cross-sectional areas, moments of inertia, and bending moments) derived from the bone mass image.

RESULTS

Work in our laboratory using commercial DEXA scanners has shown that bone strength defining details underlying observed changes in bone mass due to age, disuse, and microgravity are evident in the structural geometry; i.e., the quantification of bone size and shape. We have developed our theories based on analysis performed on BMD data derived from various adult populations including Russian cosmonauts. Using a conventional DEXA image as shown in Figure 1, we extract bone mineral density profiles at important skeletal locations (e.g., proximal shaft and femoral neck). Key properties measured and derived from this data include the BMD, the subperiosteal width, the section modulus (related to strength), and an estimate of cortical dimensions.

Initial results of studies on a population of adults (ages 20 to over 90) have produced the following information. On average, there was little decline in the section modulus at the femoral neck and proximal shaft through the seventh decade of life for females and the eighth decade for males. During this same life progression, the BMD in females decreased by approximately 25% (through the seventh decade) and in males by approximately 20% (through the eighth decade) from nominal young adult values. Even though the BMD decreased significantly, the maintenance of section modulus suggests that the bones became mechanically more efficient with age – due to subperiosteal expansions. Analysis of subperiosteal widths for the same patient data produced an almost linear subperiosteal width increase with increasing age. The rate of increase was about 2% per decade relative to normal young adult values. Aging causes an increase in endocortical diameter and with appropriate activity (loading), bone is added to the subperiosteal surface thus increasing its diameter. Hence, the bones get larger with thinner cortical walls, thus maintaining strength despite BMD loss. For example, a 10% loss in bone mineral density can be compensated by less than a 1 mm increase in subperiosteal diameter.

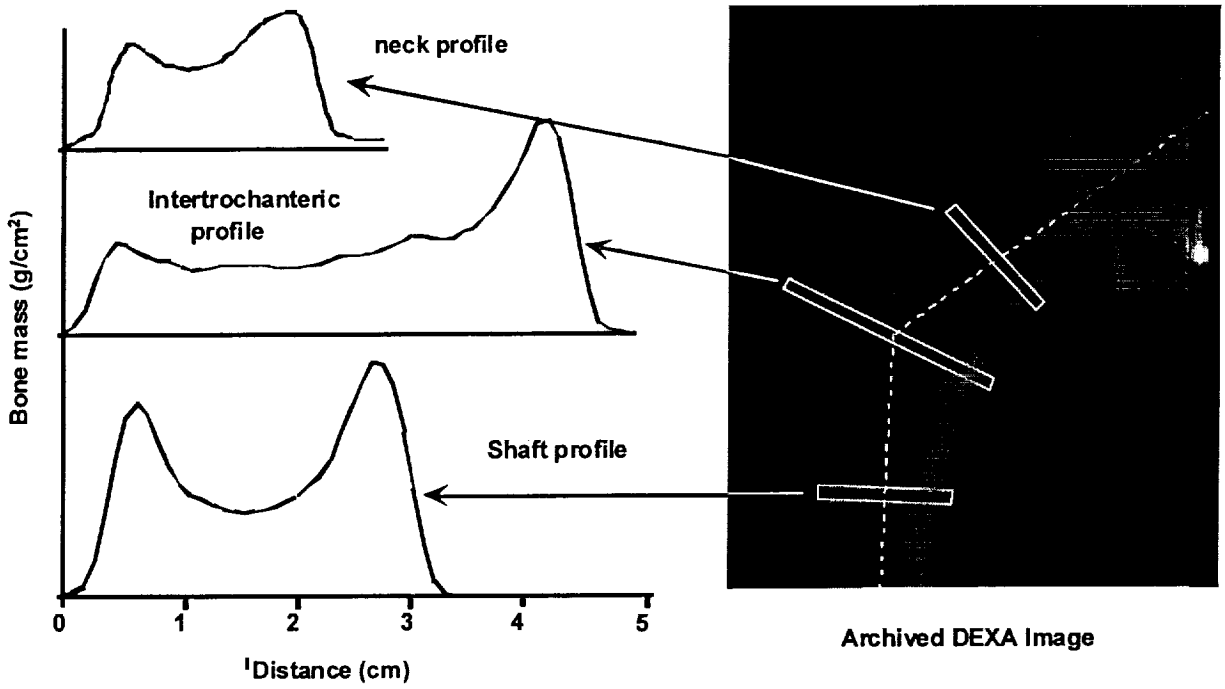


FIGURE 1. Geometry extraction from BMD DEXA image.

A preliminary examination of Russian cosmonaut data (20 individuals with an average of almost 6 months in space) produced supporting results. Since loading stimulates subperiosteal expansion, it would be expected that bone mineral density loss would not be accompanied by subperiosteal expansion and, thus, both BMD and bone strength (section modulus) in cosmonauts would be reduced. This is exactly what has been observed in the analyzed cosmonaut data. BMD decreased by 4-8%. Section modulus decreased by 4-8%, while the subperiosteal width remained relatively constant.

In a study of postmenopausal women (age 65 plus), we have compared BMD data, section modulus, and cortical dimensions for women who maintained or gained weight with those that had significant weight loss. The population with static or increasing weight had reduced BMD (nominally 1 to 2% over a four-year period) and some decline in cortical thickness. This was coupled with an offsetting increase in subperiosteal diameter, thus preserving bone strength or section modulus. In the women with significant weight loss, the BMD reduced 2 to 4% (over four years) and the decline in cortical thickness was greater than the subperiosteal diameter increase, thus causing a decline in bone strength, which, again, is consistent with our hypothesis.

While these preliminary results are quite encouraging, there are several technical issues associated with making the measurements and subsequent analysis including: (1) the structural changes are subtle and difficult to detect due to the poor image quality of current DEXAs, (2) structure measurements require high scan precision (or large numbers to compensate), and (3) reproducible positioning is critical to accurately trace structural changes with time. Because of these needs, we have embarked upon the development of an improved DEXA system as described below.

NEW INSTRUMENT DEVELOPMENT

As shown above, commercial DEXA scanners can be used to measure geometry from mass data and image dimensions but are optimized for measurement of bone mass, not geometry. Poor spatial resolution and constraints of two-dimensional projections of three-dimensional bones limit precision and accuracy of current systems. A

whole body scanning DEXA system has been designed specifically for bone geometry measurements. The scanner is based on a kV-filter switching X-ray generator and a high-resolution, large-area, flat-panel detector that uses an amorphous silicon diode array.

The design incorporates rotational capability to provide multiple projections so that complex cross-sectional geometries can be evaluated in three dimensions. Algorithms for the derivation of three-dimensional geometry from as few as three projections have been implemented. A theoretical analysis of performance indicates that bone mass precision will exceed that of commercial DEXA systems, and spatial resolution should be better than three-line pairs/mm in the patient. Scanning attributes are incorporated to make measurement precision nearly independent of patient thickness.

A full-size, bench-top test bed of the scanner has been constructed for testing of principles and experimental verification of simulations. The prototype incorporates a high-precision rotation and translation stage to provide scanning capability. The test bed, in conjunction with the high-resolution detector (1408 x 1888 active array of 127 μm square pixels) and our analysis software, has produced some exciting preliminary results. Figure 2 is a bone mineral density (BMD) image of a human femur immersed in a cylinder of water (to simulate the presence of fatty tissue). The femur had some muscle tissue attached, as can be seen in Figure 2. The same bone was imaged on a commercial DEXA scanner located at the Johns Hopkins Hospital. This scanner is typical of the current commercial state-of-the-art and is in regular service scanning patients. The BMD image output from this scanner is shown in Figure 3. The improvement in spatial and contrast resolution with our scanner is quite evident by comparing the two figures. This improvement is further elucidated by the graph in Figure 4. The curves, one from each scanner, are measured bone projected thicknesses on a slice through the shaft of the femur. The fine variations on the profile of our development scanner are not noise, but actually reflect small changes in projected bone thickness.

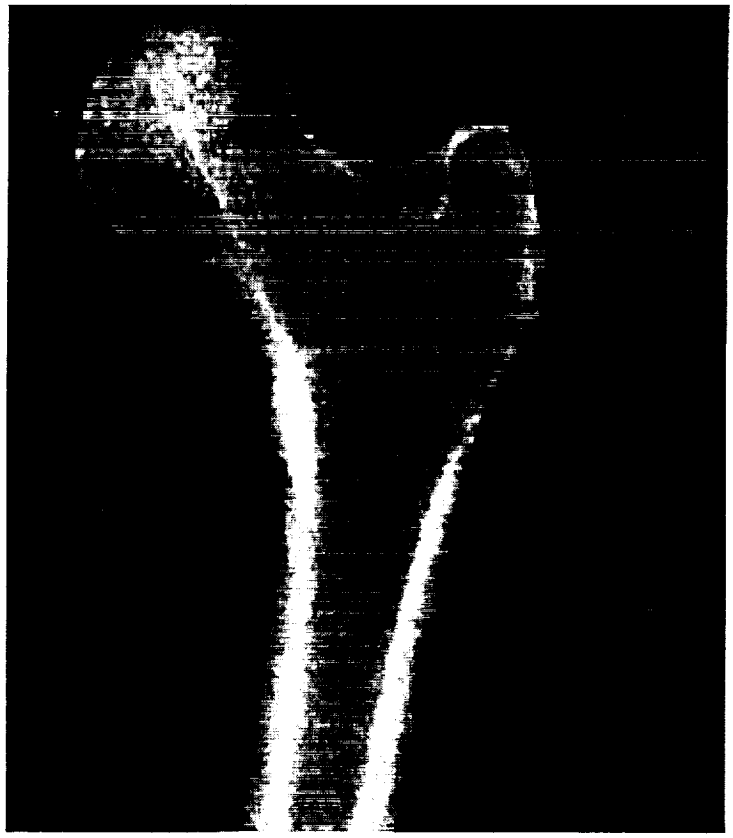


FIGURE 2. BMD image with large area detector.

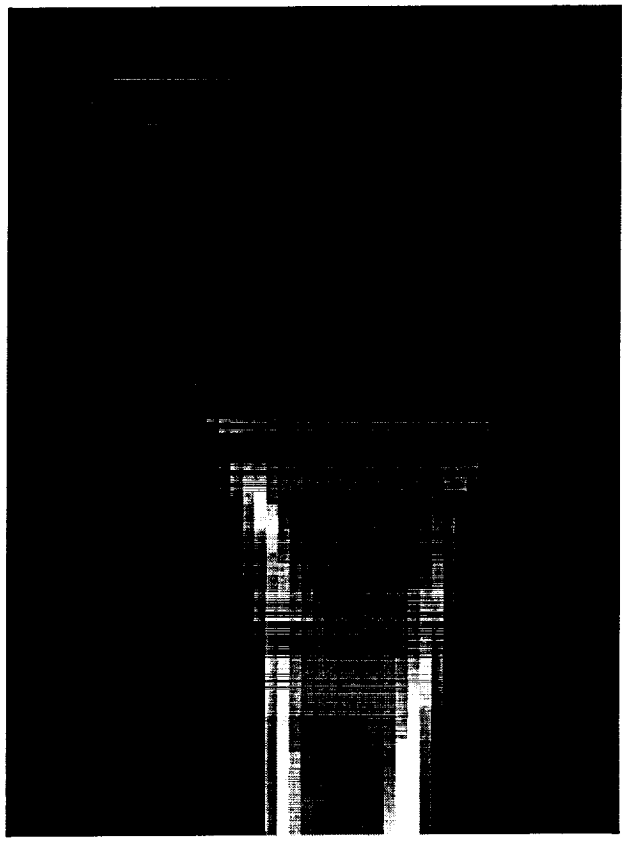


FIGURE 3. Femur BMD image, commercial DEXA.

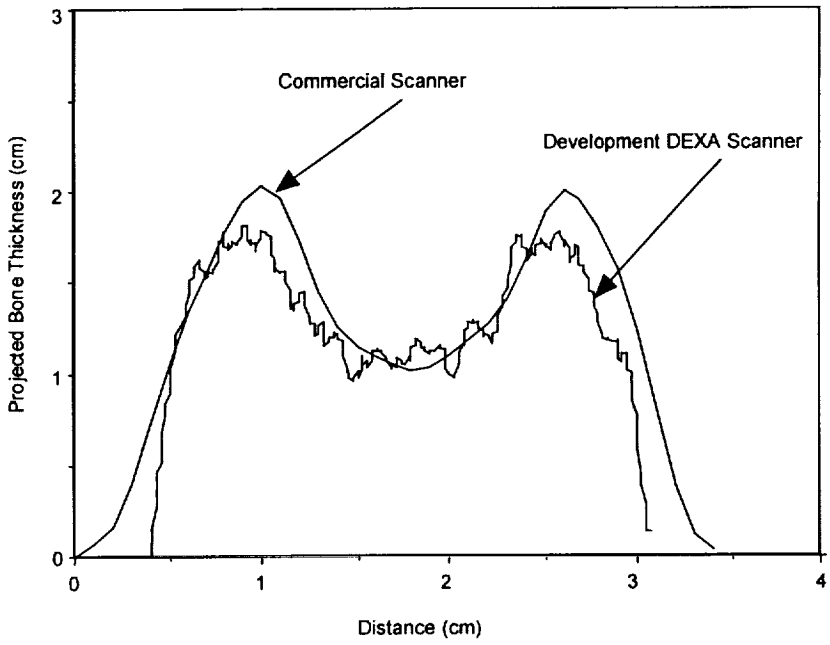


FIGURE 4. Bone thickness profiles.

SUMMARY

Analysis of DEXA data strongly suggests that bone mineral density alone is not the overall determining factor in bone strength and, hence, fracture risk. Bone strength can be maintained despite BMD loss due to structural remodeling. Gravity and weight maintenance are major elements in retaining bone strength. The precision measurement of structure is key to the understanding of the bone loss process and the subsequent prediction of fracture risk. Current instruments are inadequate for the task since structural changes are subtle and require high precision measurements. A system capable of removing patient position errors is critical. Our current high-precision, multiple projection DEXA system is being designed to capitalize on these requirements. Performance to date has been extremely encouraging.

ACKNOWLEDGEMENTS

Development of the Multiple Projection DEXA Scanning System has been funded by the National Space Biomedical Research Institute under Cooperative Agreement NCC 9-58 with the National Aeronautics and Space Administration, Lyndon B. Johnson Space Center, Houston, Texas.

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PRECISION BONE AND MUSCLE LOSS MEASUREMENTS BY ADVANCED, MULTIPLE PROJECTION DEXA (AMPDEXA) TECHNIQUES FOR SPACEFLIGHT APPLICATIONS

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INTRODUCTION

The purpose of the Advanced, Multiple Projection, Dual Energy X-ray Absorptiometry (AMPDEXA) project [1] is to design, build, and test a precision scanner for monitoring the deleterious effects of weightlessness on the human musculoskeletal system during prolonged spaceflight. The instrument uses dual energy X-ray absorptiometry (DEXA) principles and is designed to: measure bone mineral density (BMD), decompose soft tissue into fat and muscle, and derive structural properties (cross-sections, moments of inertia). Such data permits assessment of microgravity effects on bone and muscle and the associated fracture risk upon returning to planetary gravity levels. Multiple projections, coupled with axial translation, provide three-dimensional (3-D) geometric properties suitable for accurate structural analysis. This structural analysis coupled with bone models and estimated loads defines the fracture risk. The scanner will be designed to minimize volume and mass (60 kg goal), while maintaining the required mechanical stability for high-precision measurement. The AMPDEXA will be able to detect 1% changes in bone mass and geometry and 5% changes in muscle mass.

METHODS

Humans lose bone mass throughout their adult life and within any given population age group there are a significant number of men and women with low BMD; yet, most osteoporotic fractures occur at the end of life. This phenomena is usually attributed to factors such as propensity for falls, reduced bone quality, etc., but we strongly believe the explanation lies in the bone's structural details. Our hypothesis is that throughout most of adult life, aging bones become more structurally efficient and retain their strength even though BMD declines. The homeostatic mechanism for strength maintenance depends on skeletal loading. Thus, to maintain bone strength, normal loading on the skeletal system must be maintained. Absence of loading during prolonged spaceflight (or disuse) can cause uncompensated loss of bone strength. Even reduced loading (caused by muscle wasting and inactivity in the elderly) can cause a disruption in the bone strength maintenance mechanism.

Current bone and muscle mass measurements (via conventional DEXA or ultrasound) are regional averages that obscure structural details. Since the mechanical consequences of lost bone and muscle are reflected in the structure, an absolute determination of skeletal mechanical competence is needed to supplement the loss measurements. Engineering properties of the bones can be derived from DEXA-generated BMD data [2]. The method derives geometrical measurements from the BMD images. From such images, we extract BMD profiles at important skeletal locations (e.g., proximal shaft and femoral neck). Key properties measured and derived from these profiles include the BMD, the subperiosteal width, the section modulus (related to strength), and the cortical dimensions.

RESULTS

Initial adult population (ages 20 to over 90) studies have produced the following information. On average, there was little decline in the section modulus at the femoral neck and proximal shaft through the seventh decade of life for females and the eighth decade for males. Over the same age range, the BMD in females decreased by approximately 25% (through the seventh decade) and in males by approximately 20% (through the eighth decade). Even though the BMD decreased significantly, the maintenance of section modulus suggests that the bones became mechanically more efficient with age – due to subperiosteal expansions. Analysis of subperiosteal widths produced an almost linear subperiosteal width increase with increasing age. The rate of increase was about 2% per decade relative to nominal young adult values. Aging causes an increase in endocortical diameter and with appropriate activity (loading), bone is added to the subperiosteal surface. Hence, the bones get larger with thinner cortical walls, thus maintaining strength despite BMD loss. For example, a 10% loss in BMD can be compensated by less than a 1 mm increase in subperiosteal diameter.

Russian cosmonaut data (20 individuals with 6 months average space time) produced supporting results. Since loading stimulates subperiosteal expansion, it was expected that BMD loss would not be accompanied by subperiosteal expansion and, thus, both BMD and bone strength (section modulus) would be reduced. This is exactly what the cosmonaut data shows. BMD decreased by 4-8%. Section modulus decreased by 4-8%, while the subperiosteal width remained relatively constant.

In a study of postmenopausal women (age 65 plus), we have compared BMD data, section modulus, and cortical dimensions for women who maintained or gained weight with those that had significant weight loss. Women with static (increasing) weight had reduced BMD (nominally 1 to 2% over a four-year period) and some decline in cortical thickness, but there was an offsetting increase in subperiosteal diameter, thus preserving bone strength or section modulus. In women with significant weight loss, the BMD reduced 2 to 4% (over four years) and the decline in cortical thickness was greater than the subperiosteal diameter increase, thus causing a decline in bone strength, which, again, is consistent with our hypothesis.

The preliminary results are quite encouraging, but there are several important technical issues: (1) the structural changes are subtle and difficult to detect due to the poor image quality of current DEXAs, (2) structure measurements require high scan precision (or large numbers to compensate), and (3) reproducible positioning is critical to accurately trace structural changes with time. The AMPDEXA system under development addresses these three issues.

NEW INSTRUMENT DEVELOPMENT

Commercial DEXA scanners measure BMD, not geometry. Poor spatial resolution and the two-dimensional nature of a single projection limit system precision and accuracy. A whole body AMPDEXA system has been designed specifically for bone geometry measurements. The scanner is based on a kV-filter switching X-ray generator and a high-resolution, large-area, flat-panel amorphous silicon detector. The design incorporates multiple projection capability so that complex, 3-D cross-sectional geometries can be evaluated. Algorithms for the derivation of 3-D geometry from as few as three projections have been implemented. A performance analysis indicates that BMD precision will exceed that of commercial DEXA systems with a spatial

resolution better than three-line pairs/mm. Scanning attributes are incorporated to make measurement precision nearly independent of patient thickness.

A full-size, test bed has been constructed to verify principles and simulation results. Scanning is provided by high-precision rotation and translating stages. The test bed, in conjunction with a high-resolution detector and our analysis software, has produced some exciting preliminary results. Figure 1(a) is a BMD image of a human femur immersed in a cylinder of water (simulates fatty tissue). The same bone was imaged on a new commercial DEXA scanner located at the Johns Hopkins Hospital as shown in Figure 1(b). The improvement in spatial and contrast resolution with our scanner is quite evident by comparing the two figures. This improvement is further elucidated by the graph in Figure 1(c). The curves are measured bone projected thicknesses on a slice through the femoral shaft. The fine variations on the AMPDEXA profile are not noise, but reflect small changes in the actual bone thickness.

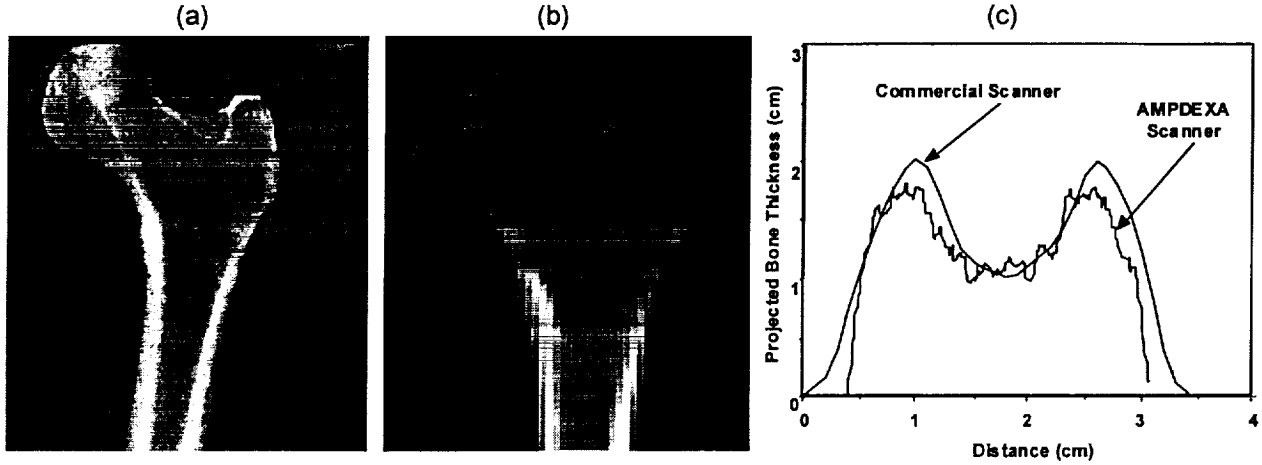


Figure 1. Comparison AMPDEXA versus conventional DEXA. (a) AMPDEXA BMD image. (b) Commercial DEXA BMD image (same bone as (a)). (c) Bone thickness profiles.

SUMMARY

DEXA data analysis strongly suggests that BMD alone is not the overall determining factor in bone strength and, hence, fracture risk. Bone strength can be maintained despite BMD loss due to structural remodeling. Gravity and weight maintenance are major elements in retaining bone strength. Precision structural measurement is key to the understanding of the bone loss process and fracture risk prediction. Current instruments are inadequate for the task since structural changes are subtle and require high precision measurements. A system capable of removing patient position errors is critical. Our AMPDEXA system is being designed to capitalize on these requirements. Performance to date has been extremely encouraging.

REFERENCES

1. The AMPDEXA System is funded by the National Space Biomedical Research Institute, Cooperative Agreement NCC 9-58 with the NASA/Johnson Space Center, Houston, Texas.

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Advanced Multiple Projection DEXA (AMPDEXA) Scanner for Precision Bone and Muscle Loss Measurements

H. K. Charles, Jr., Thomas J. Beck, Howard S. Feldmesser,
Thomas C. Magee, Thomas C. Spisz, and Vincent L. Pisacane

Abstract

Bone structural information derived from dual energy x-ray absorptiometry (DEXA) data is shown to be relevant in explaining bone mineral density (BMD) loss versus strength-related observations in both aging populations and individuals exposed to microgravity for prolonged periods. Commercial instruments are not optimized to make these critical structural measurements. Progress on the development of an advanced multiple projection DEXA (AMPDEXA) scanner for making precision bone and muscle loss measurements and accessing their resulting implications for bone strength and fracture risk is described.

Humans lose bone mass throughout their adult life and within any given age group there are many men and women with low BMD; yet, most osteoporostic bone fractures occur at end of life. Most researchers attribute this to factors such as falls, reduced bone quality, etc., but we believe the explanation lies in the bone's structural. Our methods rest on the hypothesis that aging bones become more structurally efficient and retain their strength even though BMD declines. Strength retention is based on a compensatory subperiosteal expansion of the bone generated by skeletal loading. Absence of loading due to prolonged spaceflight or muscle wasting and inactivity in the elderly can cause uncompensated loss of bone strength.

Results using the AMPDEXA system will be described, linking bone structure changes and mechanical properties to bone strength and reduced BMD in both aging and astronaut populations. The number of x-ray projections required to produce accurate structural information will be presented, along with techniques to eliminate repeatability artifacts due to patient position.

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IEEE Engineering in Medicine and Biology Society

23-28 July 2000
Chicago, IL

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

Project Title: *Instrumentation For Non-Invasive Assessment Of Cardiovascular Regulation*

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EXECUTIVE SUMMARY

It is critically important to be able to assess alterations in cardiovascular regulation during and after space flight. We developed instrumentation for the non-invasive assessment of such alterations that can be used on the ground and potentially during space flight. This instrumentation will be used by the Cardiovascular Alterations Team at multiple sites for the study of the effects of space flight on the cardiovascular system and the evaluation of countermeasures. In particular, the Cardiovascular Alterations Team anticipates using this instrumentation in conjunction with ground-based human bed-rest studies and during application of acute stresses *e.g.*, tilt, lower body negative pressure, and exercise. In addition, the Cardiovascular Alterations Team anticipates using this instrumentation to study astronauts before and after space flight and ultimately, during space flight. The instrumentation may also be used by investigators in other physiologic areas related to space flight, such as neurovestibular, human performance, chronobiology, and psycho-social behavioral, to measure changes in autonomic nervous function.

The instrumentation is based on a powerful new technology – cardiovascular system identification (CSI) – which has been developed in our laboratory. CSI provides a non-invasive approach for the study of alterations in cardiovascular regulation. This approach involves the analysis of second-to-second fluctuations in physiologic signals such as heart rate and non-invasively measured arterial blood pressure in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of multiple physiologic mechanisms, CSI provides a closed-loop model of the cardiovascular regulatory state in an individual subject.

Until now application of CSI currently required off-line computerized analysis of recorded physiologic signals by an expert user. The user interacted iteratively with the computer to preprocess the data, select data segments for analysis, run the CSI analyses, and evaluate and interpret the results. Thus the availability of this technology was limited to highly expert users located in Professor Cohen's laboratory. In this project, we developed integrated instrumentation capable of acquiring the physiologic signals, performing the CSI analysis in a fully automated fashion, and displaying the results on-line. The design of this instrumentation will be such that users with minimal training (including astronauts and other NSBRI investigators) can perform CSI onsite, conveniently and effectively.

The availability of this instrumentation is essential for effectively studying the cardiovascular effects of space flight and for the subsequent development and evaluation of appropriate countermeasures. In particular this instrumentation will be used by the Cardiovascular Alterations Team in the study and development of countermeasures to the development of post-flight orthostatic hypotension. The development of such instrumentation may also have significant clinical impact on the diagnosis and treatment of patients with a variety of cardiovascular and neurological disorders.

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PROJECT RESEARCH ACTIVITY

We have previously demonstrated the efficacy of a CSI method for the non-invasive assessment of alterations in cardiovascular regulation. However, the application of this method currently required off-line analysis by an expert user, which limited its utility essentially to the laboratory of the principal investigator. We hypothesized that we could develop an automated, on-line CSI instrument that could be applied by a user with minimal training to study effectively the alterations in cardiovascular regulation resulting from space flight. Thus, the objective of this project was to develop an instrument for the non-invasive assessment of alterations in cardiovascular regulation for use on the ground and potentially during space flight by an individual with minimal training.

We implemented the software in the Windows NT 4.0 environment using the C++ programming language. Windows NT was chosen because of its widespread use and because of its user friendly interface. C++ is an object oriented language which will allow for rapid development of efficient software. Many of the algorithms were developed using MATLAB software which was then compiled into C++ code.

We utilized the following hardware system

- Pentium II 300 MHz Thinkmate computer, SCSI hard disk, 21 inch monitor, Diamond Viper Graphics Card
- Tektronix Phaser 560 Color Laser Printer
- Keithley DAS-1701ST analog to digital converter and data acquisition card.
- Fujitsu DynaMO 640 Optical Drive

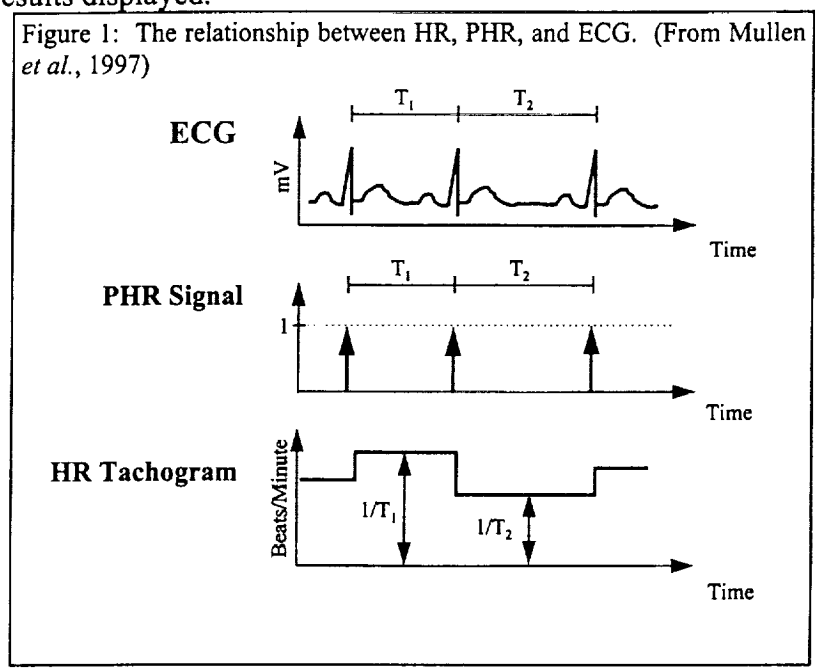
There are two modes of analysis for this instrumentation, on-line and off-line. In the on-line analysis mode, the data is read from the A/D board (which is acquiring the physiological signals) and is displayed on the screen in real time. The unprocessed input data is also stored on optical disk in real time. Thus, when in on-line analysis mode, the system performs the tasks of signal acquisition, display, and storage in addition to the main CSI analysis functions. During on-line analysis, the operator is presented with a panel which allows him or her to access functions such as: start recording, stop recording, pause recording, and change settings. Some examples of available settings include: specifying which input signals correspond to which channels, the sampling rates of the channels, and the magnification of the display.

The results of CSI are shown on the left side of the window which is updated every time new results are calculated. By default, new CSI parameters are calculated every few seconds and are based on the previous 6 minutes of data received through the A/D board. Either of these time lengths may be changed through the Settings interface. The format of

the CSI output presentation has been developed. It includes plots of the four impulse responses, shown on the left-hand side of Figure 2, as well as the Fourier transforms of N_{HR}, N_{ABP}, and ILV.

The real time processing of signals is achieved as follows. Within a second after the signal is read from the A/D board and displayed on the screen, a peak detector annotates QRS complexes from the incoming ECG signal. This allows the derivation of the Pulsatile Heart Rate PHR signal (Figure 1) which is used for CSI. The next stage of processing is done when the time interval to perform the next CSI calculation has been reached. At this point, signals are downsampled to 90 Hz and 1.5 Hz with an appropriate FIR antialiasing filter, preparing them for system identification procedures. Next, the main CSI is carried out and the results displayed.

Figure 1: The relationship between HR, PHR, and ECG. (From Mullen *et al.*, 1997)



Previously, CSI analysis involved first collecting the data and then processing the data and running the system identification algorithms off-line. We designed computationally efficient algorithms to perform CSI on-line, making the results available to the operator automatically and within a few minutes of the start of the procedure.

The second mode of operation is off-line. In this case, signals are read from disk and are displayed on the screen for the user to interactively review forward or backward. The off-line analysis will have some extra functions compared to the on-line analysis, which can be accessed through menus in addition to the panel. In off-line analysis, a section of data can be annotated and then a specific type of analysis can be performed and the results stored.

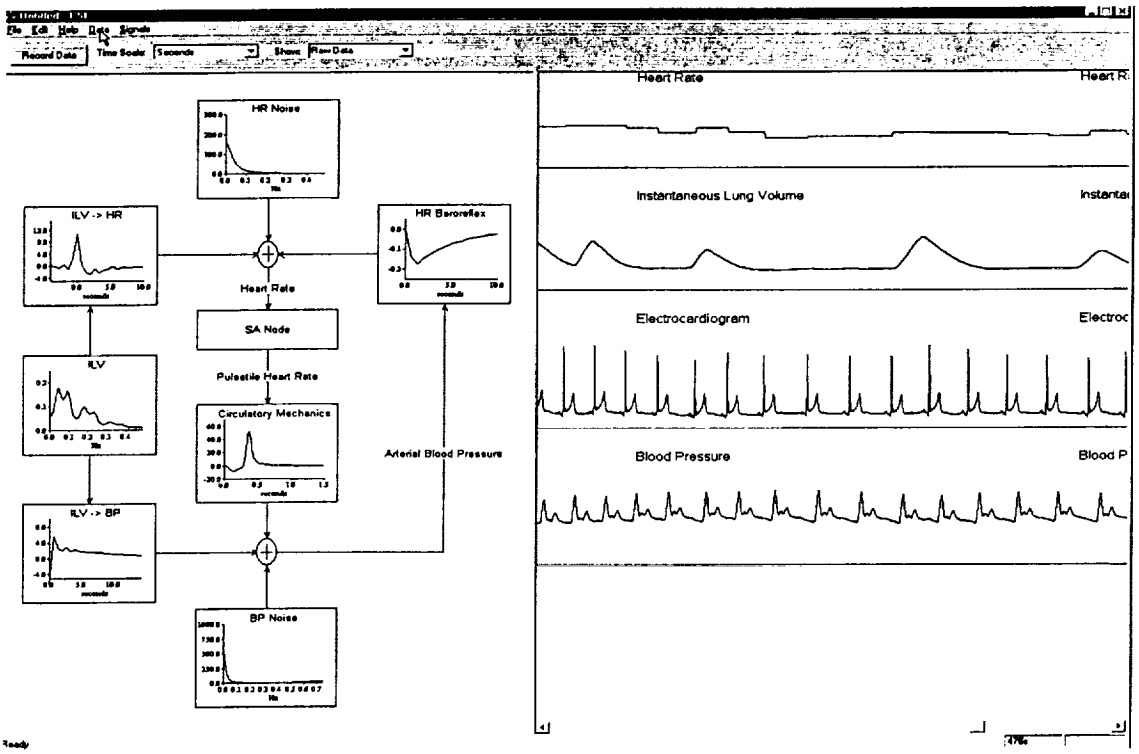
The application of our original CSI method required the interaction of an expert user for some of the data processing. For example, user interaction is required for ECG peak detection, calibration, and selection of a specific segment of data for identification (*e.g.*, selection of a data segment free of motion artifact, other data 'glitches', arrhythmias, etc.). Many of these features have now been automated, specifically ECG peak detection,

calibration, deglitching. Data segment selection has been partially automated through the use of trend plot analysis.

CSI analysis is subject to artifactual errors due to the presence of noise, motion artifact and missed or falsely detected beats. We have implemented trend plot displays of the CSI parameters (such as peak amplitude, area, and characteristic times of the impulse response functions) and the power spectra (total power, low frequency power, and high frequency power) [Mullen *et al.*, 1997]. Visual inspection of these trend plots enable the user to identify data segments in which CSI results are consistent over time; artifact tends to cause abrupt aberrations in the values of the CSI parameters.

The basic system and was demonstrated during the February 1999 NSBRI external advisory committee meeting hosted by the Applied Physics Laboratory at Johns Hopkins.

Figure 2. Screen Shot of the signal display window CSI program running in off-line analysis mode. The acquired signals are shown on the right, while the derived transfer functions are shown on the left.



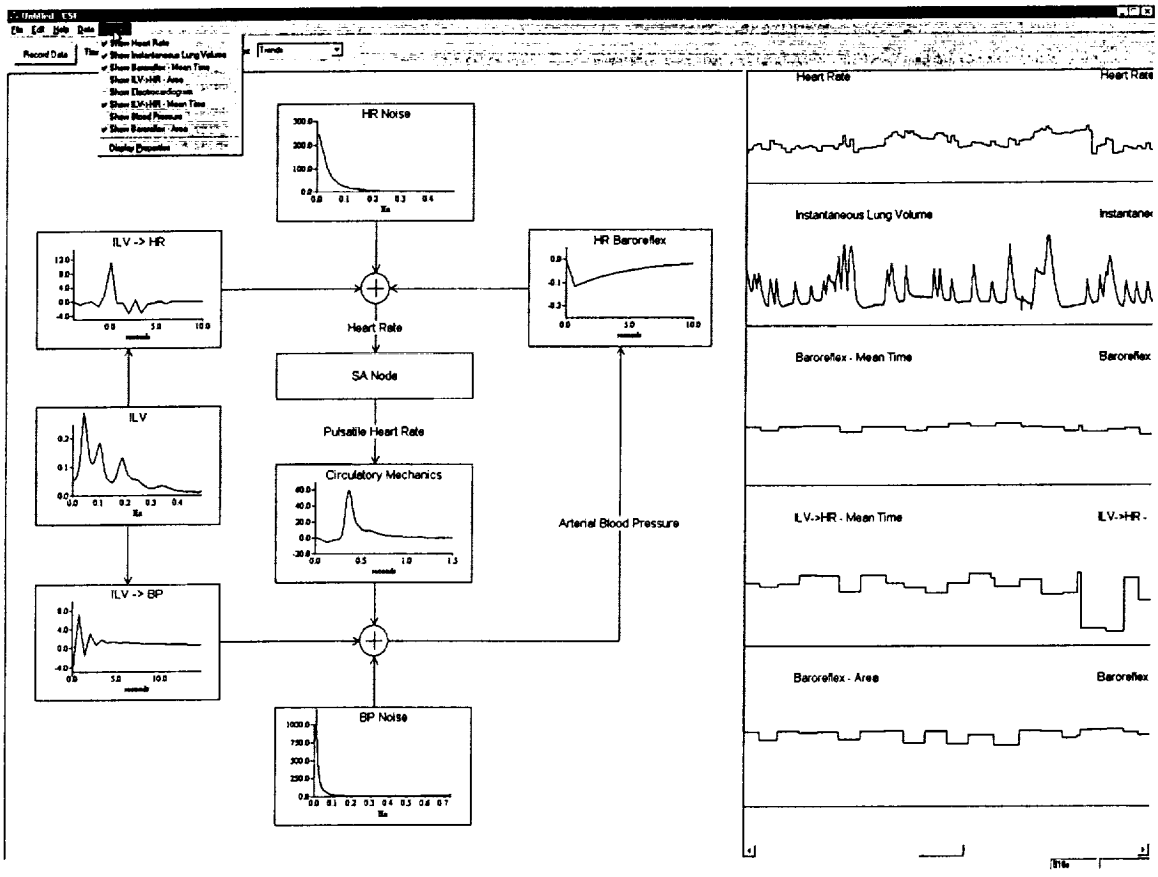
A screen shot of the main window of the program is shown in Figure 2. The program can be run in two main modes: on-line and off-line analysis. During online analysis, the program will automatically display both the physiological signals (right-hand side of Figure 2), the CSI model (left-hand side of Figure 2. The operator has the option of

scrolling through the entire data collection history, reviewing the data history including the CSI model parameters, concurrent with data collection. Thus, data acquisition and data analysis may be performed simultaneously.

During offline analysis, the operator has the option to run detailed analyses and annotate the data.

Additional software features have been completed including signal calibration capabilities and some noise correction algorithms such as "deglitching" of input signals.

Figure 3. Screen Shot of the signal display window CSI program showing trend plots calculated from transfer functions.



To facilitate non-expert analysis, we have increased the "user friendliness'' of the program reducing data collection to a single "point-and-click'' operation.

Related Studies

CSI during Motion Sickness

We conducted a parallel study to determine if CSI measures of autonomic function are alterned during maneuvers that induce motion sickness (Mullen et al, 1998). In 18 subjects, motion sickness was induced using sinusoidal rotation and the use of left-right visual field inversion while the subject performed manual tasks. The subjects were brought to the point where they developed moderate motion sickness. The transfer function from instantaneous lung volume to heart rate was used as the measure of autonomic tone. No change in this transfer function was measured in these studies. In contrast, simple maneuvers such as change in posture cause large and easily measurable changes in this transfer function. These findings do not support the notion moderate motion sickness is manifested as a generalized autonomic response.

CSI in Patients with Diabetic Autonomic Neuropathy

We studied 60 patients with diabetic autonomic neuropathy and 37 control subjects. Patients were classified on the basis of standard autonomic testing as having minimal (no measurable defect compared to controls), moderate or severe autonomic neuropathy. The 60 patients and 37 control subjects also underwent CSI testing. Quantitative analysis of the parameterized CSI impulse response functions and the power spectra of the noise sources, revealed that patients with increasing degrees of autonomic neuropathy had diminished amplitude of the autonomically mediated impulse response functions while the mechanically mediated impulse response functions were not significantly changed. CSI detected a statistically significant difference in the autonomically mediated impulse response functions between the minimal neuropathy group and controls whereas standard autonomic testing did not.

IMPLICATIONS FOR FUTURE RESEARCH

CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate, arterial blood pressure (ABP), and instantaneous lung volume (ILV – respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of all the physiologic mechanisms coupling these signals, CSI provides a model of the closed-loop cardiovascular regulatory state in an individual subject (see left-hand side of Figures 2 and 3). The model includes quantitative descriptions of the heart rate baroreflex as well as other important physiologic mechanisms. With an additional non-invasive measurement of stroke volume (SV – ultrasound Doppler method), the model may be extended to also include the characterization of the peripheral resistance baroreflex – which may play a central role in the development of orthostatic intolerance – and measures of systolic and diastolic function. The development of user friendly instrumentation for the non-invasive measurement of CSI will permit the wide application of this technology to solve problems related to spaceflight and as well as the diagnosis and management of a variety of diseases on earth.

We have developed a prototype system for real time CSI analysis. The basic system requires further field testing. The system would benefit from further improvements in the real-time preprocessing algorithms, interfacing to Analog-to-Digital conversion system,

improvement of display and interface functions, provision for data and file management, and improved digital filtering algorithms.

Other modifications that would benefit the system include advancing automated noise identification and correction algorithms, optimization of system identification algorithms, implementation of augmented system identification models, analysis and presentation of system identification parameterizations and final implementation of user-friendly interface and help features.

We would like to be able to incorporate stroke volume measurements into our CSI model. However, reliable measurement of stroke volume using Doppler ultrasound is a technically challenging procedure. As an alternative to Doppler ultrasound, we preliminarily examined other non-invasive methods for determining cardiac output. These alternative methods would derive the cardiac output from the more readily obtainable signals (ABP, ILV, heart rate) already being collected [e.g. see Wesseling, *et al.*, 1993].

NSBRI Benefits

This project primarily benefits the Cardiovascular Alterations Team. In addition, the Neurovestibular Adaptation and Human Performance Factors, Sleep and Chronobiology have expressed interest in using the instrumentation for measurement of autonomic function in their human studies. This instrumentation may also be used in the long term bed-rest studies of the Bone Demineralization/Calcium Team to measure changes in cardiovascular regulation.

NASA Benefits

The availability of this instrumentation will make it possible to non-invasively measure alterations in closed-loop cardiovascular regulation resulting from real or simulated spaceflight. This is critically important to address the problem of development of postflight orthostatic hypotension. The CSI approach has been successfully used by the Cardiovascular Alterations Team to identify mechanisms leading to the development of orthostatic hypotension and to develop an effective pharmacologic countermeasure, midodrine.

Earth Benefits

Alterations in cardiovascular regulation occur in a wide range of disease processes including diabetes mellitus, heart failure, hypertension, etc. The instrumentation being developed here will permit the non-expert to assess quantitatively alterations in closed-loop cardiovascular regulation in patients.

Literature Citations

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Mullen TJ, Berger Rd, Oman CM and Cohen RJ. Human Heart Rate Variability Relation is Unchanged During Motion Sickness. Journal of Vestibular Research 1998;8(1):95-105.

Wessling KH, Jansen JRC, Settels JJ, Screuder JJ. Computation of aortic flow from pressure in humans using a non-linear, three-element model. J. Appl. Physiol. 74(5): 2566-2573, 1993

APPENDIX A – Project Research Data

Contained in this report and in publications listed in Appendix B

APPENDIX B – Publications

Mullen TJ, Berger Rd, Oman CM and Cohen RJ. Human Heart Rate Variability Relation is Unchanged During Motion Sickness. Journal of Vestibular Research 1998;8(1):95-105.

Mukkamala R, Mathias JM, Mullen TJ, Cohen RJ and Freeman R. System Identification of Closed-Loop Cardiovascular Control Mechanisms: Diabetic Autonomic Neuropathy. Am. J. Physiol. 1999;276 (Regulatory Integrative Comp Physiol. 45):R905-912.

Cohen RJ, Iyengar N, Mullen TJ, Ramsdell CD, Sundby G. User Friendly Instrumentation for Non-Invasivc Assessment of Alterations of Cardiovascular Regulation Associated with Space Flight. Proceedings. First Biennial Space Biomedical Investigators' Workshop, January 11-13, 1999, League City, Texas pp. 570-572.

Chen, Chun-Cheng. Automated Cardiovascular System Identification. Master of Engineering in Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 2000.

APPENDIX C - Publications Enclosed

Mullen TJ, Berger Rd, Oman CM and Cohen RJ. Human Heart Rate Variability Relation is Unchanged During Motion Sickness. Journal of Vestibular Research 1998;8(1):95-105.

Mukkamala R, Mathias JM, Mullen TJ, Cohen RJ and Freeman R. System Identification of Closed-Loop Cardiovascular Control Mechanisms: Diabetic Autonomic Neuropathy. Am. J. Physiol. 1999;276 (Regulatory Integrative Comp Physiol. 45):R905-912.

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Chen, Chun-Cheng. Automated Cardiovascular System Identification. Master of Engineering in Electrical Engineering and Computer Science, Massachusetts Institute of Technology, 2000.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Project Report

September 2000

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MINIATURE TIME-OF-FLIGHT MASS SPECTROMETER

EXECUTIVE SUMMARY

(1) Key Findings

Major advances must occur to protect astronauts from prolonged periods in near-zero gravity and high radiation associated with extended space travel. The dangers of living in space must be thoroughly understood and methods developed to reverse those effects that cannot be avoided. Six of the seven research teams established by the National Space Biomedical Research Institute (NSBRI) are studying biomedical factors for prolonged space travel to deliver effective countermeasures. To develop effective countermeasures, each of these teams require identification of and quantitation of complex pharmacological, hormonal, and growth factor compounds (biomarkers) in humans and in experimental animals to develop an in-depth knowledge of the physiological changes associated with space travel.

At present, identification of each biomarker requires a separate protocol. Many of these procedures are complicated and the identification of each biomarker requires a separate protocol and associated laboratory equipment. To carry all of this equipment and chemicals on a spacecraft would require a complex clinical laboratory, and it would occupy much of the astronaut's time. What is needed is a small, efficient, broadband medical diagnostic instrument to rapidly identify important biomarkers for human space exploration.

The Miniature Time-Of-Flight Mass Spectrometer Project in the Technology Development Team is developing a small, high resolution, time-of-flight mass spectrometer (TOFMS) to quantitatively measure biomarkers for human space exploration. Virtues of the JHU/APL TOFMS technologies reside in the promise for a small (less than one cubic ft), lightweight (less than 5 kg), low-power (less than 50 watts), rugged device that can be used continuously with advanced signal processing diagnostics. To date, we have demonstrated mass capability resolution from under 100 to beyond 10,000 atomic mass units (amu) in a very small, low-power prototype for biological analysis. Further, the electronic nature of the TOFMS output makes it ideal for rapid telemetry to earth for in-depth analysis by ground support teams.

A major objective of this project was the design and development of a mass spectrometer *system architecture* that can be utilized for diagnostics based on complex, non-volatile biomarkers species. Because this requires multiple (and generally incompatible) ionization sources, we have designed, built and tested an orthogonal extraction time-of-flight (TOF) mass spectrometer analyzer that incorporates a dual matrix-assisted laser desorption/ionization (MALDI) and electron ionization (EI) source.

The orthogonal extraction time-of-flight instrument was successfully completed and demonstrated. This novel instrument greatly expands the spectrum of biomarkers that can be measured by incorporating the capability of electron impact ionization with the previously demonstrated MALDI measurements. This new capability allows

measurement of substances ranging from low molecular volatile organic compounds to high molecular weight biological compounds such as proteins and carbohydrates.

The TOFMS Team has completed initial laboratory studies with critical biomarkers identified by the Muscle Alterations and Atrophy Team. The TOFMS Team has recorded full spectrum mass spectral signature of key target biomarker analytes using the MALDI technique at physiological concentrations found in urine. Sampling from urine has been chosen as a high priority for this project. Compounds investigated included: insulin-like growth factors (IGF-I), Urinary 3-methylhistidine, and estradiol. IGF-I is a potent anabolic factor that mimics most of the growth promoting actions of GH *in vivo*. IGF-1 has also been identified by the Bone Demineralization / Calcium Metabolism Team as an important biomarker.

Another biomarker identified by Muscle Alterations and Atrophy Team is urinary 3-methylhistidine. It is a measure of myofibrillar protein degradation. 3-methylhistidine cannot be re-utilized by the body. It is rapidly and quantitatively excreted in the urine. Estradiol is a steroid hormone important for the maintenance of muscle mass and bone density. It is widely speculated that steroid hormones such as estradiol play a central role in the early stages of muscle atrophy and bone demineralization.

The TOFMS team has also used matrix-assisted laser desorption mass spectrometry as a tool to *quantitatively* measure 3-MH in biological fluids. The TOFMS team analyzed various concentrations of 3-methylhistidine in water and in urine to determine the relationship between analyte concentration and analyte molecular ion intensity. The concentrations used in this study were based on 3-methylhistidine concentration typically found in urine, i.e. 20pmole – 3.5nmole. The team examined the utility of two types of internal standards, histidine, a structural analogue, and d_3-3-methylhistidine, a stable-isotope labeled analogue. 3-Methylhistidine (3-MH) samples in water and urine were prepared ranging from 5uM – 10mM, keeping the (3MH)/(histidine) ratio constant at 1:10. Protonated molecular ions for 3MH and histidine could be identified in the corresponding MALDI spectra. A plot of the ratio of relative peak intensities of (3MH)/(d_3-3-MH) versus 3-MH concentration gave a linear response with a correlation coefficient, $R^2 = 0.9799$ and a relative standard deviation of the slope of 4.00%.

The TOFMS Team has also completed initial laboratory studies with biomarkers specific to the Bone Demineralization / Calcium Metabolism Team. These include trivalent hydroxypyridinium crosslinks and creatinine. Trivalent hydroxypyridinium crosslinks are released into the circulation during bone resorption and are excreted as free pyridinolines molecules. In bone and cartilage, the collagen is bound by pyridinoline or deoxypyridinoline crosslinks. Deoxypyridinoline is found exclusively in bone while pyridinoline is found in skin, joint and cartilage. Creatinine is used to extrapolate the status of bone remodeling activity in various metabolic bone conditions.

The TOFMS Team has performed a mass spectral analysis of alendronate to determine the mass spectral pattern by MALDI and to add the compound to our library of critical biomarkers. Bisphosphonate administration to the hindlimb of suspended rats and limb immobilization studies in dogs suggest that this compound is an effective countermeasure to bone loss. Alendronate is a member of the bisphosphonate family of

drugs used to treat/prevent osteoporosis. We analyzed a commercially available product, Fosamax.

The TOFMS team has also used a breath monitoring system to examine human subjects in order to select molecules that may serve as biomarkers of normal and abnormal physiology. These molecules will be used to direct the selection of molecules to be monitored with the time-of-flight miniature mass spectrometer.

One of the objectives of the NSBRI Human Performance Factors, Sleep and Chronobiology Team is to develop strategies to monitor the circadian physiology of astronauts during long-duration space missions. The Team has identified that there is a critical need for in-flight assessment of melatonin levels. Melatonin is recognized as a very reliable marker of the human circadian pacemaker. Recent studies have indicated that there is very reliable correlation between the salivary and plasma levels. Because the sampling of plasma melatonin is an invasive procedure, it would be desirable to have a means of measuring salivary melatonin in subjects on long-duration space missions. We have performed a preliminary analysis of salivary melatonin using MALDI time-of-flight mass spectrometry of melatonin in saliva. Mass spectrometry may provide a reliable, convenient, and economical way to track melatonin during space missions.

Whole blood is the biological fluid of choice for therapeutic drug monitoring and for performing pharmacokinetic studies. Spectra for whole blood were recorded in DHB matrix and in cyano-4-hydroxycinnamic acid matrix. These spectra exhibited well-defined peaks from 100 to 400 mass units.

The risks to personnel in space from the naturally occurring radiation are generally considered to be one of the most serious limitations to human space missions. The NSBRI is examining the consequences of radiation in space *in vivo* in order to develop countermeasures, both physical and pharmaceutical, to reduce the risks of cancer and other diseases associated with such exposures. The consequences of exposure to radiation in space are considered a major limiting factor for long-duration interplanetary space travel for humans. Radiation doses in space may be hundreds of times greater than those experienced on earth. These energetically charged particles can kill cells in the body or cause mutations that may lead to cancer, cataracts, central nervous system damage or other diseases.

The TOFMS Team evaluated three novel peptide cancer biomarkers to demonstrate the utility of MALDI-TOF as a tool for the early detection of carcinomas. The advantage in using it for detection over these other methods is the robust nature of the analyzer. MALDI-TOF mass spectrometry is rapid, sensitive, and tolerant of salts in biological samples.

(2) Summary of Satisfaction of Objectives

The first-year objectives were satisfactorily completed. In year one of the program the team prioritized the biological analytes that we would investigate. We recorded mass spectral signature data of initial biomarkers. We developed a quantification protocol to

obtain biomarkers from blood, urine and breath. We coordinated TOFMS Development with other space experiments and devised a concept for a reflectron-TOFMS.

The second-year objectives were satisfactorily completed. The tasks accomplished in year 2 were to: build and test an electrospray sample deposition apparatus for quantitative analysis of biomarkers; evaluate and examine surfaces for sample inlet system; establish a quantitative method using isotopically labeled internal standards for specific biomarkers; develop a concept of operation for space-based processing of biomarkers from urine and blood; test the concept of operation on tabletop commercial TOFMS and DARPA "prototype" TOFMS; and, develop an orthogonal extraction TOFMS design for enhanced ion collection efficiency.

The third-year objectives were satisfactorily completed. The tasks were to conduct subsystems integration; interface the system for interconnectivity and interoperability; determine TOFMS functionality; evaluate selected analytes provided by other collaborators on the TOFMS; and, evaluate analytical processing methods.

(3) Implications of this Project for Risk Reduction Related to the Critical Path

The long-term implications of this ground-based research and technology development project are to lay the scientific and engineering foundations to design, build and launch a flight-qualified "Miniature Time-of-Flight Mass Spectrometer" (TOFMS) for use on space platforms such as the Space Shuttle and the International Space Station (ISS). Successful deployment of this instrument in near-earth missions will lay medial, engineering and scientific groundwork to adopt this medical diagnostic instrument for a mission to Mars later this century.

The development of the "Miniature Time-of-Flight Mass Spectrometer" will provide NSBRI/NASA with a complete medical diagnostic system to measure bone, internal organs, and soft tissues, routinely and non-invasively. This compact medical diagnostic system will provide autonomous and semi-autonomous patient monitoring systems with low false positive alarm rates.

This research project supports the goals of the Life Sciences Division of NASA to aid in the exploration of the solar system, support the achievement of routine space travel, and enrich life on Earth through the use of space technology and the application of biomedical knowledge. This research project falls into Category 4: Clinical Research in Support of Space Missions (Medicine in Extreme Environments).

The Countermeasure Readiness Level (CRL) developed by NASA describes the level of scientific maturity of applied research from the development of a hypothesis to validated procedure ready for operational implementation of procedures and devices. This scale has been developed as a method to mitigate the deleterious effects on humans engaged in space flight. Using this scale as a metric, this project was at level 5, "Proof of concept testing and initial demonstration of feasibility and efficacy." Based on the results that we have achieved to date, we believe that this project can successfully transition to countermeasurement development.

MINIATURE TIME-OF-FLIGHT MASS SPECTROMETER

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MINIATURE TIME-OF-FLIGHT MASS SPECTROMETER

I. PROJECT RESEARCH ACTIVITY

Objectives

The objectives of this program are to adapt the DARPA miniature time-of-flight mass spectrometer design for long duration human space flight; to identify important biomarkers that can be measured using this instrument; to develop a sampling system for the TOFMS tailored to analyze biomarkers in urine, blood and/or breath; and to work with the other teams in the NSBRI to integrate this spectrometer into their research protocols to deliver appropriate countermeasures.

The TOFMS technologies at JHU/APL will be adapted from a qualitative design to a quantitative sampling design for the analysis of biochemical markers in space.[1] This capability is important for space physiology sampling (urine, blood, breath) requirements. It is conceivable that successful completion of this project will yield rapid results, which traditionally take days or weeks to accomplish, when the proper TOFMS system is developed. In addition to instrument development, an important objective of this project is sampling, separation and quantitation of the target analytes.

Specific Project Aims

The overall aim of the Miniature Time-of-Flight Mass Spectrometer Project is to design, develop, and test a laboratory demonstration system capable of monitoring and analyzing compounds of biological and chemical origin in the spacecraft habitat. The engineering model will demonstrate that a very capable Time-of-Flight Mass Spectrometer (TOFMS) can be embodied in less than a 1-cu ft^3 volume, weigh less than 5 kg, and require less than 50 watts of power. It will be capable of analyzing body fluids and it will help to analyze spacecraft environment and space bioreactor products.

Specific project aims for the JHU/APL team are to *identify* and *quantify* priority biomarkers that appear in urine. Sampling from urine will be emphasized initially, because urine contains several important biomarkers and spectra of urine present less background clutter compared to a sample of blood. Biomarkers for the Muscle Alterations and Atrophy Team that can be studied in urine include growth hormone, 3-methylhistidine and ubiquitin. Biomarkers found in urine for the Bone Demineralization/ Calcium Metabolism Team includes creatinine, steroids, and deoxypridinoline crosslinks. These biomarkers are being studied at physiological concentrations in urine.

The JHU/APL team is building a library of mass spectrums of key target molecules that are found in urine. Spectra are being recorded from a commercial Kratos IV TOF Instrument and compared to an engineering model miniature time-of-flight mass spectrometer at JHU/APL. These initial results will be used to design and build a MALDI sampling and inlet system for quantitative analysis.

The JHU/APL team will down-select from the various requirements to one or two focused designs for the next generation TOFMS for space application. This decision will be based on considerations of urine sampling, the classes of biomarkers selected, the technical feasibility, cost, operational concept, user interface, and signal processing decision aids.

The JHU/APL team has initiated interactions with other NASA initiatives to assure that, if appropriate, the development of the TOFMS can accommodate additional requirements from other NASA/JSC initiatives such as the Space Bioreactor. The detection of airborne bacteria (e.g., staphylococcus sp.), fungi (e.g., Aspergillus sp.) are of priority interest. The estimate their frequency of occurrence and likely measurable concentrations need to be determined.

Background and Significance

Mass spectrometry is an extremely high-resolution technique for determining the masses of molecules and specific fragmentation products formed during vaporization and ionization. From detailed analysis of the mass distribution of the molecule and its fragments, molecular identification is accomplished. These molecular measurements can be carried out at the attomole (10^{-18} mole) level of material using specialized laboratory-based instruments. The combination of specific molecular identification and extreme sensitivity makes mass spectrometry one of the most powerful analytical laboratory tools yet developed for detection and identification of chemical and biological substances.[2]

APL Engineering Model Mass Spectrometer System

Equipment used for high-performance mass spectrometry has previously been large, heavy, and power-hungry, precluding its use in remote field measurements. More recently, the JHU/APL collaborative program has developed a "time-of-flight" (TOF) mass spectrometer, shown in Figure 1. It uses new techniques for ion formation and energy focusing, new sampling and ionization schemes, and new analysis techniques. It also incorporates advances in signal processing, high-speed digitizing electronics, fast-pulsed lasers, and miniaturized vacuum pump designs.

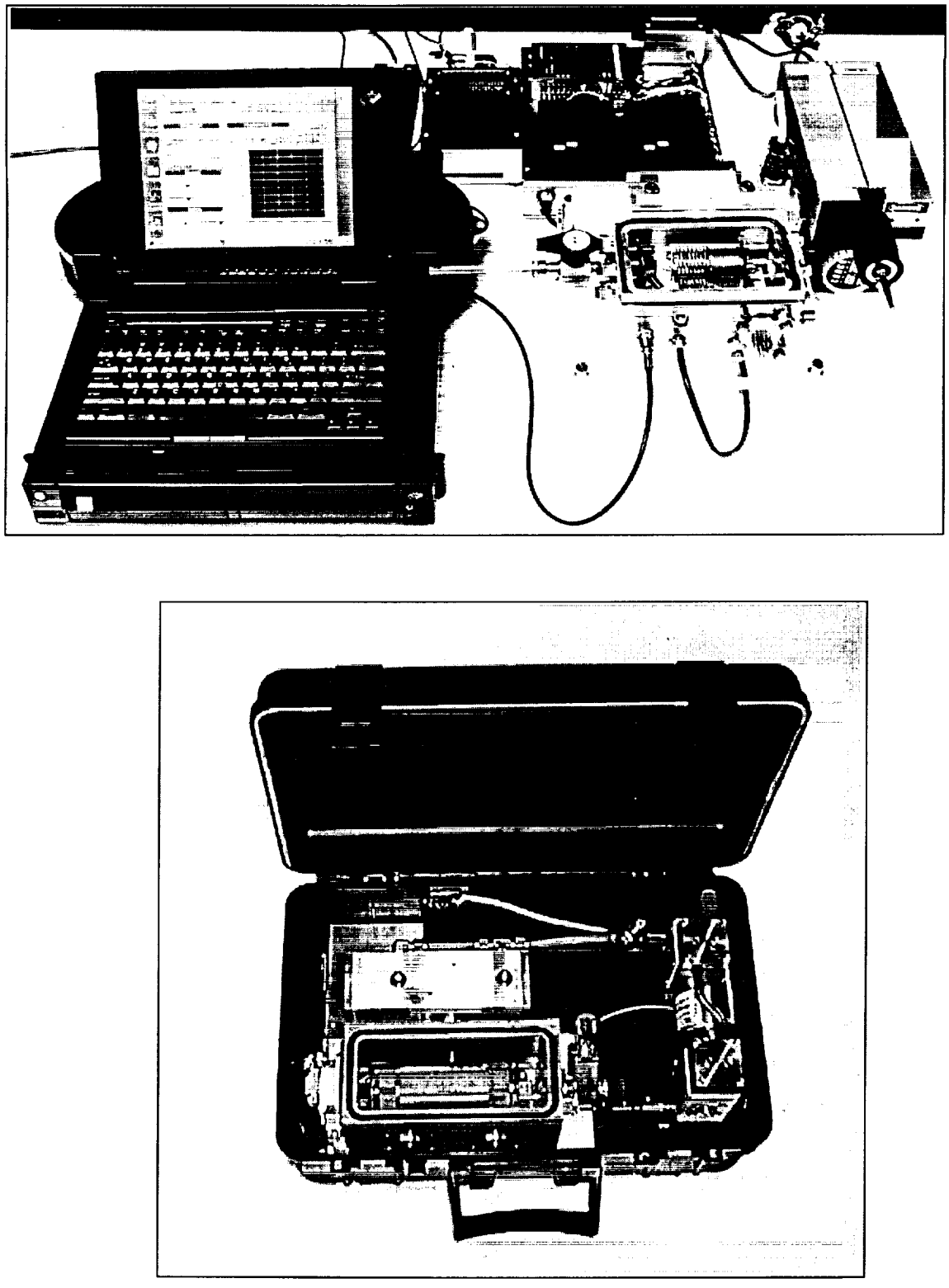


Figure 1. JHU/APL "time-of-flight" (TOF) mass spectrometer: figure (top) shows components of the instrument and figure (bottom) shows model fits in a small travel case.

This spectrometer has a coaxial design. Ions are created and accelerated in the source region, allowed to drift across a field-free region, and caused to enter an ion mirror that reflects the ions back toward the channel electron multiplier detector. In developing the TOF mass spectrometer, APL used realistic electrostatic modeling to carefully tailor ion creation in the source region and employed advanced, ion reflectron-based kinetic energy correction schemes.

Principle of Operation

The TOF mass spectrometer has long been considered an excellent candidate for field-portable operation owing to its ruggedness, intrinsic low weight, and simplicity of operation. Consider the linear TOF mass spectrometer schematically depicted in Figure 2.

Ions are formed in a short, evacuated source region generally delimited by a backing plate and an extraction grid. A voltage V placed on the backing plate imposes an electric field (E-V/s) across the source region, which accelerates all of the ions to the same kinetic energy, where m is the mass of the ion, v is its velocity, e is the charge on an electron, and z is the number of charges on the ion. As the ions pass through the extraction grid, their velocities depend inversely upon the square root of their mass. The ions then pass through a much longer drift region to the detector, where they are measured as a TOF spectrum. The TOF spectrum can be converted directly to a mass spectrum using known values of the accelerating voltage and drift length.

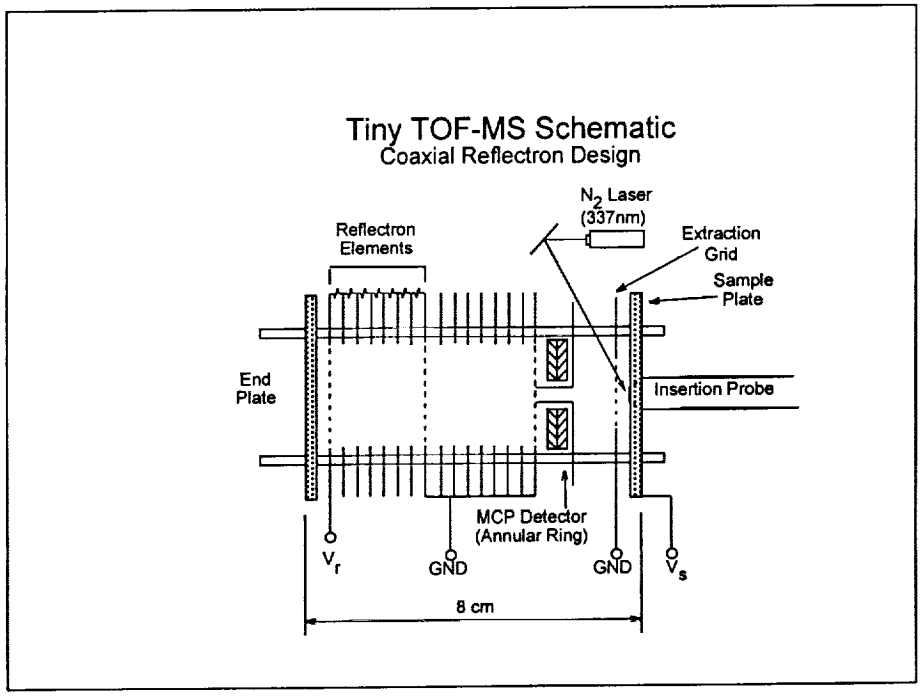


Figure 2. Schematic of linear TOF mass spectrometer

When ions of the same mass are formed in different locations in the extraction field, ions near the back of the source will be accelerated to a higher kinetic energy than those closer to the extraction grid. Hence, ions of the same mass can enter the drift region with different velocities, with the faster ions arriving at the detector before the slower ions.

Analyzing Samples Using Laser Desorption Techniques

When a sample is placed on a conducting surface that is in contact with an accelerating potential and is exposed to laser irradiation of sufficient power density, ions are formed and accelerated into a mass spectrometer for analysis. Depending on the peak power density of the laser, different ionization and molecular fragmentation conditions are obtained. Karas et al.[3] and Tanaka et al.[4] introduced matrix-assisted laser desorption/ionization (MALDI) using lower laser irradiances (typically 1 MW/cm^2). With this technique, biomolecules as large as 300,000 Daltons can be ionized and desorbed intact into the gas phase for mass analysis. The basis of MALDI is the interaction of a pulsed laser beam with a laser-absorbing matrix material into which analyte molecules are dispersed. Pulsed laser energy is absorbed by the matrix and transferred to the analyte, causing it to be ionized and desorbed into the gas phase. In the process, the analyte chemically interacts with fragment ions of the matrix, forming molecular adduct ions.

The MALDI process generally involves wet chemical techniques, whereby a solution of the matrix molecule is physically mixed with a solution containing the analyte. The resulting mixture is applied to a sample probe, allowed to dry and introduced into the mass spectrometer for analysis. We are developing an alternative concept for MALDI processing in which a "test strip" containing internal standards will be incorporated with the matrix material. A sample of urine or blood will be drawn onto the test strip by capillary action. The test strip may contain a mirodialysis membrane to further separate component compounds in urine. The test strip will also serve as a substrate that can be directly inserted into the vacuum of the TOFMS for analysis.

Biomarker Detection, Identification and Quantification

The identification of clinical biomarkers and the development of countermeasures are critical to support human space exploration and research. Clinical practice requires time-consuming analysis of target molecules to confirm a condition or disease exists and it can lead to a delay in treatment or lead to over-prescription of a countermeasure for treatment of a condition that does not exist. Conventional techniques for biomarker identification rely upon large clinical facilities containing a suite of instrumentation and wet chemistry laboratories. Diagnosis is time consuming and requires a highly trained technical staff.

A flow diagram for using JHU/APL Tiny TOF-MS in human space exploration to detect biomarkers is shown in Figure 3.

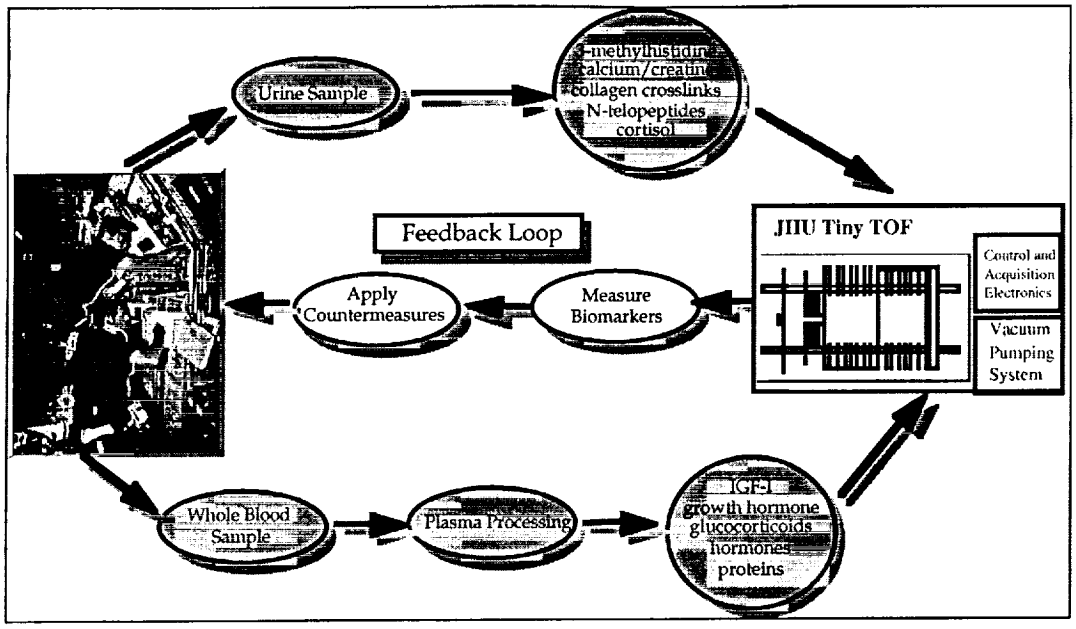
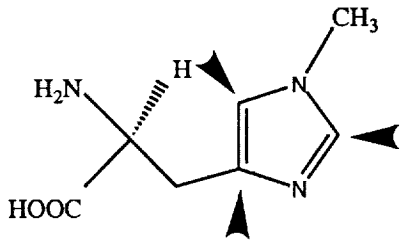


Figure 3. Tiny TOF-MS in human space exploration to detect biomarkers

From a single sample of urine or blood several biomarkers can be identified and quantitatively measured in a matter of minutes. Initially, the project is concentrating on detecting biomarkers from urine. However, blood analysis will be added to the program after a complete protocol for urine is established. From a sample of urine or blood several biomarkers can be identified and appropriate countermeasures can be applied. The JHU/APL Tiny TOF-MS can also be used to measure the effectiveness of applied countermeasures.

Direct quantitation by MALDI -TOF MS will eliminate the need for chromatographic sample analysis. In this project, quantitation of biomarkers is be performed by using isotopically labeled internal standards when available. For example, 3-Methylhistidine can be prepared with one or more isotopically labeled carbon atoms. The unlabeled and labeled compounds have indistinguishable chemical properties once incorporated into the matrix, except for the fact that the two compounds have different formula weights. The concentration of a biomarker can be determined if the concentration of the labeled compound in the matrix is known. A linear response of $[M+H]^+$ to biomarker concentration should be observed. The arrows in Chemical Structure I point to labeled carbon atoms in the ring moiety of the 3-Methylhistidine compound.



Chemical Structure I: 3-Methylhistidine. Arrows point to istopically labeled carbons in the histidine ring.

For unknown analytes, other internal biological molecules having similar chemical structures found in blood and urine samples are being investigated to obtain quantitation.[567]

Modifications to the Original Proposal and Rationale for Modifications
Upon selection of this project, the plan was modified to increase collaboration between this project and the other NSBRI Teams to develop TOFMS system sampling, ionization, and analysis tools for high priority biomarkers. Also given a high priority is quantitation methods and sample preparation procedures which will lead to focused development of an engineering model TOFMS system for long-duration space flight. The plan was also modified to decrease the emphasis on evaluating identify priority airborne and liquid toxic chemicals such as ammonia, hydrazine, and formaldehyde which can be identified by other methods.[8]

TOFMS Team Collaborations
(1) Established collaboration with Professor Robert Schwartz at Baylor College of Medicine to integrate TOFMS development into Muscle Alterations and Atrophy Project. Dr. Schwartz has identified critical biomarkers to evaluate which are important to his NSBRI research program.

(2) TOFMS team obtained important biomarker information from Professor Jay R. Shapiro at Johns Hopkins School of Medicine. (Collaboration between TOFMS Team and Bone Demineralization / Calcium Metabolism Team has identified critical biomarkers to evaluate which are important to his NSBRI research program. Dr. Shapiro has assisted the TOFMS team in developing protocols for measuring biomarkers in whole blood.

(3) JHU/APL team is working with MIMS Technology Co. to investigate their membrane inlet system. Initial laboratory studies demonstrate that biomarkers can be detected using MIMS technology. Work is ongoing to select a membrane material for use with urine.

(4) TOFMS team has held discussions with Perkin Elmer Corporation regarding collaboration on a medical diagnostic system based on TOFMS technology.

II. RESULTS AND ACCOMPLISHMENTS

(Results summarized in each section below support a specific NSBRI objective or technology requirement.)

A. Design and Development of an Orthogonal Extraction Time-of-Flight Mass Spectrometer System

A major objective of this project was the design and development of a mass spectrometer *system architecture* that can be utilized for *diagnostics* based on complex, non-volatile biomarkers species. Because this requires multiple (and generally incompatible) ionization sources, we have designed, built and tested an orthogonal extraction *time-of-flight* (TOF) mass spectrometer analyzer that incorporates a dual *matrix-assisted laser desorption/ionization* (MALDI) and *electron ionization* (EI) source. The design and development of a mass spectrometer for the NSBRI effort is supported jointly by a Defense Advanced Research Projects Agency (DARPA) grant.

The basic design, shown in figure 4, incorporates the following features:

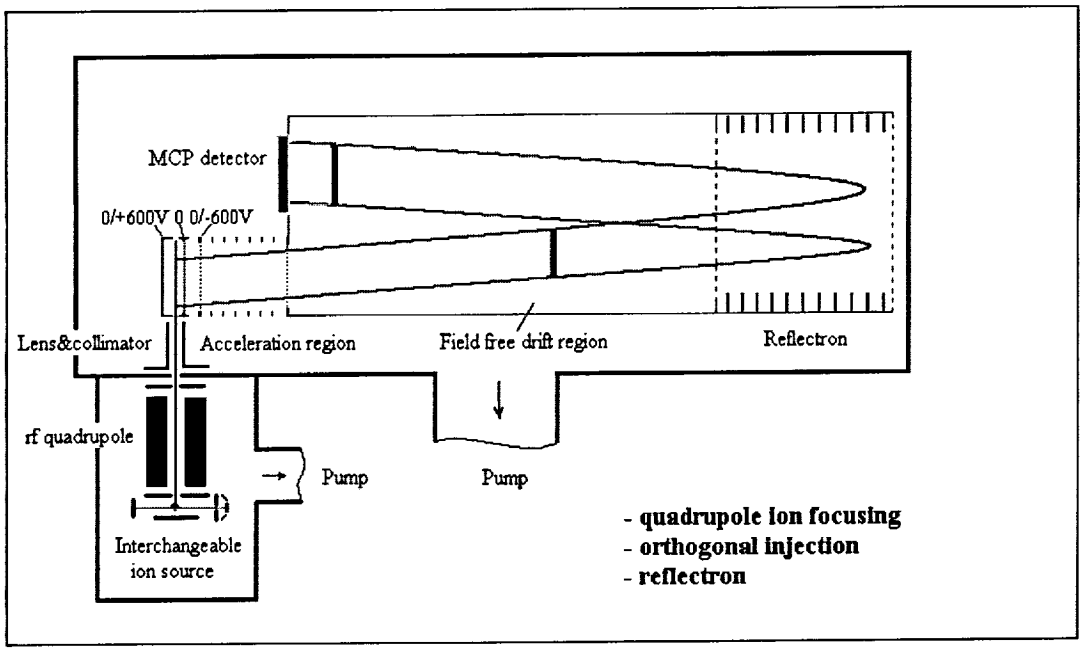


Figure 4. Schematic Diagram of the Orthogonal Extraction Time-of-Flight Mass Spectrometer System

Multiplex recording advantage. Because the TOF mass spectrometer records all ions simultaneously (without scanning), it is capable of monitoring several diagnostic biomarkers simultaneously and with high sensitivity.

Orthogonal Extraction. Time-of-flight mass analyzers have traditionally been regarded as low mass resolution instruments. The loss of mass resolution is in fact the result of several *initial conditions* during the time of ionization that include temporal, spatial and kinetic energy (velocity) distributions. Approaches that have been used to improve mass resolution include the use of *reflectrons* to correct the kinetic energy spread, *time-delayed extraction* to compensate at the source for differences in kinetic energy, and *orthogonal extraction,* which focuses ions along a direction perpendicular to the velocity distribution. In the simplest orthogonal extraction scheme, ions from the source (having different initial kinetic energies and velocities) are focused and collimated along a single direction and into a storage region by a combination of electrostatic lenses and orifices. Ions are then pulsed from the storage region by an extraction field that provides space focusing to the detector, or to the focal point of a reflectron.

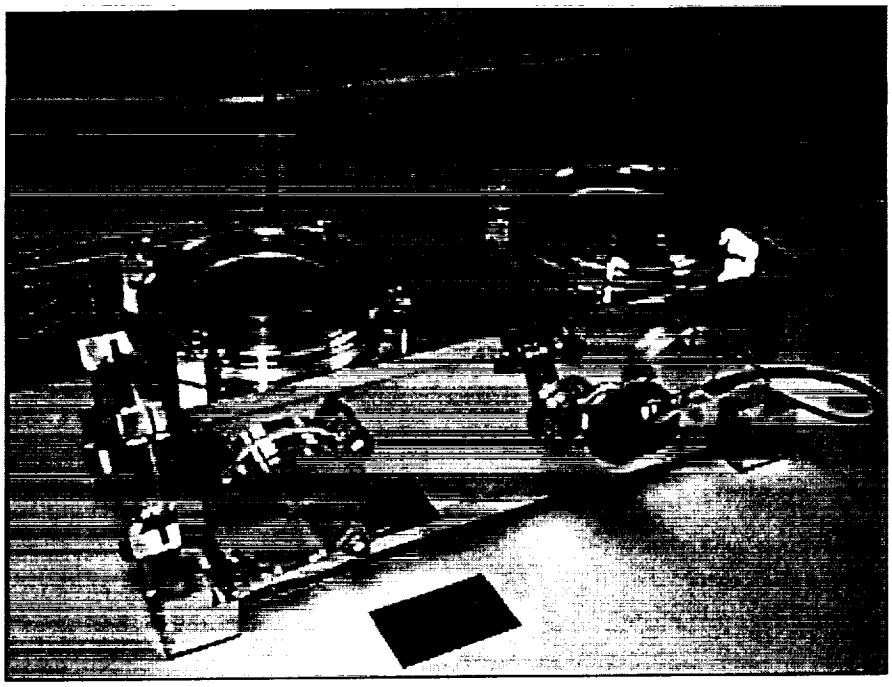
Quadrupole Injection. The use of a RF-only quadrupole enhances the performance of orthogonal extraction. It improves sensitivity because ions are collimated by the RF field rather than by restriction by an orifice, thereby admitting a larger portion of the initial ion beam into the storage area. Additionally, the RF-only quadrupole collimates ions even when there are large numbers of collisions resulting from the use of a high-pressure source, and indeed can utilize background gases to collisionally reduce the kinetic energy distribution.

Reflectron. While orthogonal extraction can improve mass resolution by focusing ions at the detector, better resolution can be achieved when it is used in combination with a reflectron. In this case, the extraction field focuses the ions at the focal point of the detector.

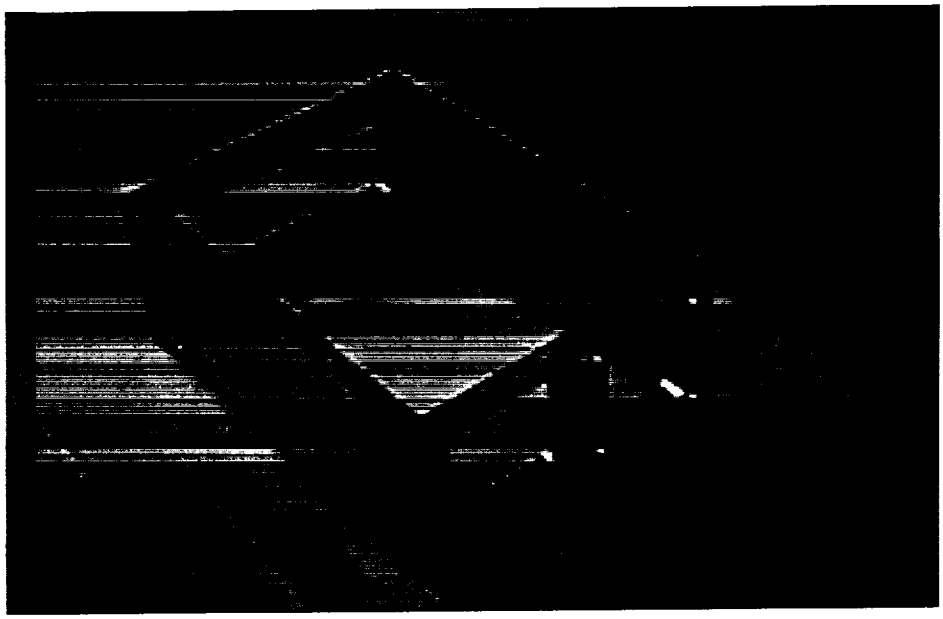
Electron Ionization (EI). Electron ionization (70 eV) is used for the analysis of volatile or chemically-derivatized biomarkers from urine, blood or other fluids.

Matrix-assisted Laser Desorption/Ionization (MALDI). MALDI will be used for proteomic and other non-volatile biomarkers. The instrument is equipped with a 600 ps pulsed nitrogen laser (337 nm); however, it is planned to eventually incorporate a variable wavelength IR laser system, using a miniaturized *optical parametric oscillator* (OPO) being developed in collaboration with Science & Engineering Services, Inc. (Burtonsville, MD).

Vacuum Chamber and Pumping System. The vacuum chamber was manufactured by Nor-Cal, Inc. (Yreka, CA), and is installed in a custom cabinet that also contains the system electronics. The chamber is pumped down by a Leybold (Export, PA) Turbovac 361 400-liter/second turbomolecular pump to a vacuum of 6.4×10^{-8} Torr.



Electronics, Data Acquisition and Laser. Electronics modules contain two Bertran (Hicksville, NY) 5 kV high voltage power supplies for the accelerating and reflectron voltages, and a EG&G (Oak Ridge, TN) First Flight 2Gsample/second digitizer. The laser is a Laser Photonics (Orlando, FL) LN 300 pulsed nitrogen laser.



Ion Optics. The ion optical bench for alignment of the flight tube, reflectron, ion source extraction optics, and detector has been designed and fabricated and tested. The reflectron has been constructed from lens elements produced by Kimball Physics (Wilton, NH).

In year 3 of the project, the orthogonal extraction time-of-flight instrument was successfully completed and demonstrated. This novel instrument greatly expands the spectrum of biomarkers that can be measured by incorporating the capability of electron impact ionization with the previously demonstrated MALDI measurements. This new capability allows measurement of substances ranging from low molecular volatile organic compounds to high molecular weight biological compounds such as proteins and carbohydrates. In a proposed follow-on project to NSBRI, this instrument will be interfaced to a gas chromatograph for separation of volatile and semi-volatile compounds before they are introduced into the mass spectrometer.

Figure 5 shows a spectrum of the organic compound perfluorotributylamine recorded on the Johns Hopkins University orthogonal extraction time-of-flight mass spectrometer system.

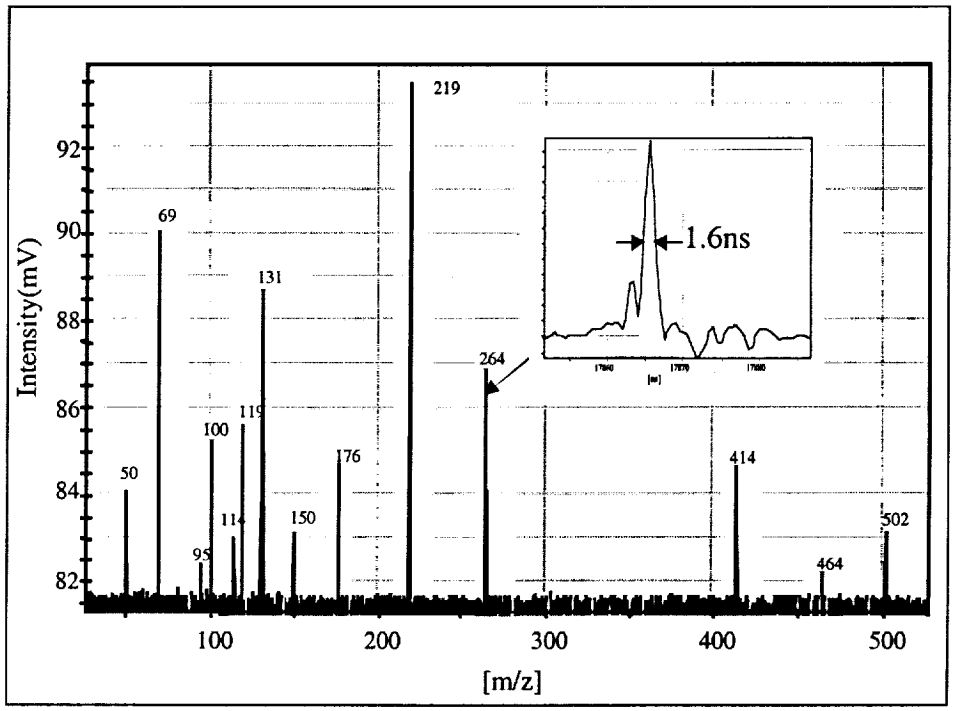


Figure 5. Perfluorotributylamine (PFTBA) or FC43 mass spectrum.

Perfluorotributylamine is used as a mass and intensity calibrant standard for mass spectrometry. This spectrum very well matches the tabulated reference spectrum, demonstrating proper operation of the instrument. Furthermore, the extremely high repetition rate (in this case 4 kHz), very high mass resolution (5500 based on FWHM for the ion at m/z=264), and excellent mass accuracy and stability (better than 100 ppm after 24 hours) indicates that this instrument has outstanding performance.

As stated above, a future goal of our development program is to couple the mass spectrometer to a miniature gas chromatograph. In that process, a carrier gas such as helium will be employed. A key factor in the applicability of the instrument to measurement of volatile biomarkers is the effect of helium gas at various background

concentrations. The performance of the instrument under these conditions is shown in Figures 6 and 7.

In the first experiment 3.5 mTorr of helium gas was introduced, along with the PFTBA sample, and spectra were acquired. See figure 3 below. In this experiment, the performance of the instrument actually increased as indicated by the narrowing of the peak at m/z=264 (higher mass resolution).

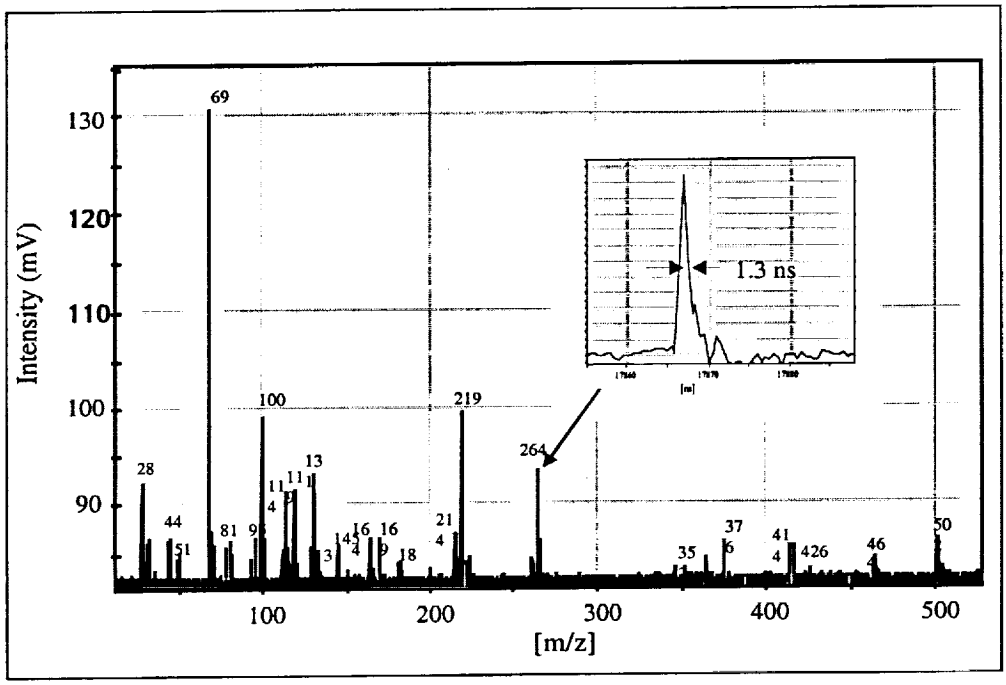


Figure 6. Mass Spectrum of PFTBA with 3.5 mTorr He.

For higher helium gas loads the performance was slightly degraded as indicated in Figure 7.

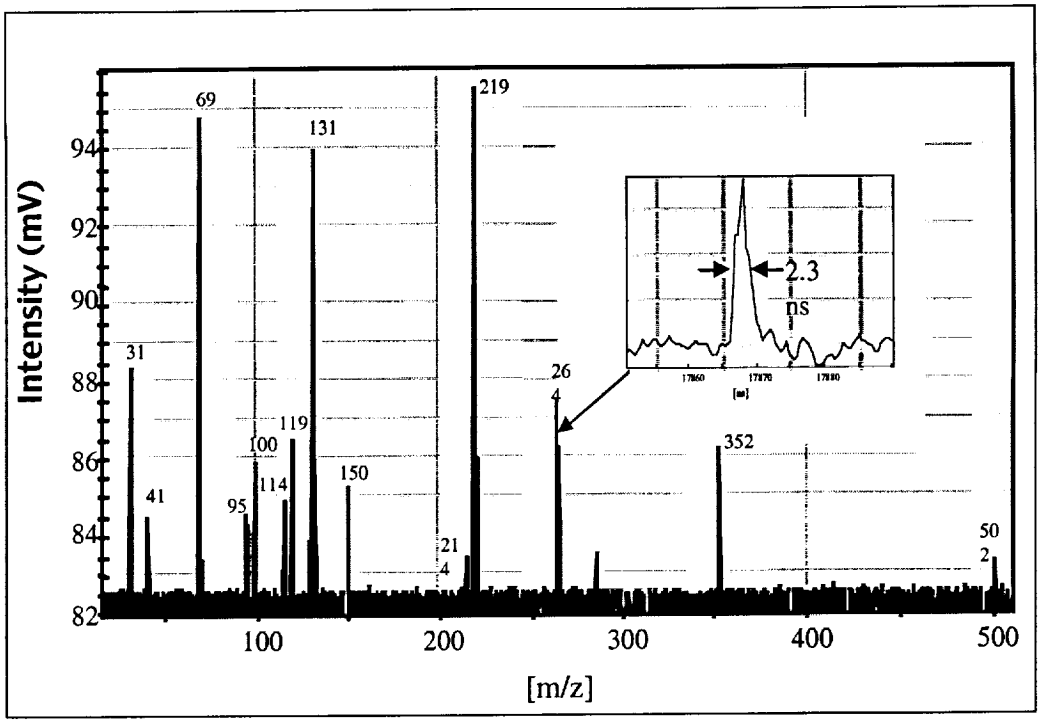


Figure 7. Mass Spectrum of PFTBA with 6.5 mTorr He

The peak at m/z=264 was broadened due to the effect of the buffer gas. This series of experiments was performed to determine the operating point for the gas flow from the gas chromatograph into the mass spectrometer. The flows and pumping rates of the interface system have been designed to provide a partial pressure of helium in the 3.5 mTorr range and the system is ready for integration.

B. Collaboration with Dr. Robert Schwartz at Baylor College of Medicine to Integrate TOFMS Development into Muscle Alterations and Atrophy Project

Exposure to reduced gravity during space travel profoundly alters the loads placed on bone and muscle. Astronauts lose muscle mass and strength while in space. Exercise countermeasures are so important that other missions for astronauts may not be given enough time and as a countermeasure is only partially effective, in remedying severe muscle atrophy. The data from humans in space indicates a very rapid atrophy of skeletal muscle. After 5-day flights, mean cross-sectional areas of muscle fibers were 11 and 24% smaller in type I and II fibers. These changes occurred even though countermeasures were undertaken by astronauts.

The Muscle Alterations and Atrophy Project has established a need for pharmacological, hormonal, and growth factor countermeasures and in-depth knowledge of molecular mechanisms. The team needs to know levels of biological compounds: including growth hormone, insulin-like growth factors (IGF-I), glucocorticoids: cortisol (which may play a central role in the early stages of muscle atrophy), and 3-methylhistidine (breakdown product of muscle proteins).

The JHU/APL team has demonstrated the technical feasibility for evaluating these biomarkers by TOFMS. Figures 4-8 give examples of spectra obtained on a MALDI-TOF testbed.

In space, growth hormone might be part of an important endocrine replacement therapy to restore muscle mass. Because the administration of either GH or IGF-I to GH-deficient animals under experimental conditions result in increased muscle and connective tissue mass, it has been proposed that these hormones may be important in maintaining muscle mass. IGF-I is a potent anabolic factor that mimics most of the growth-promoting actions of GH *in vivo*.

Figure 8 is a MALDI mass spectrum of insulin-like growth factors (IGF-I). IGF-1 is a single chain polypeptide of 70 amino acid residues cross-linked by three disulfide bridges. The concentration of the compound in solution is 1.2 pmoles. The protonated molecular ion appears at m/z= 7724.

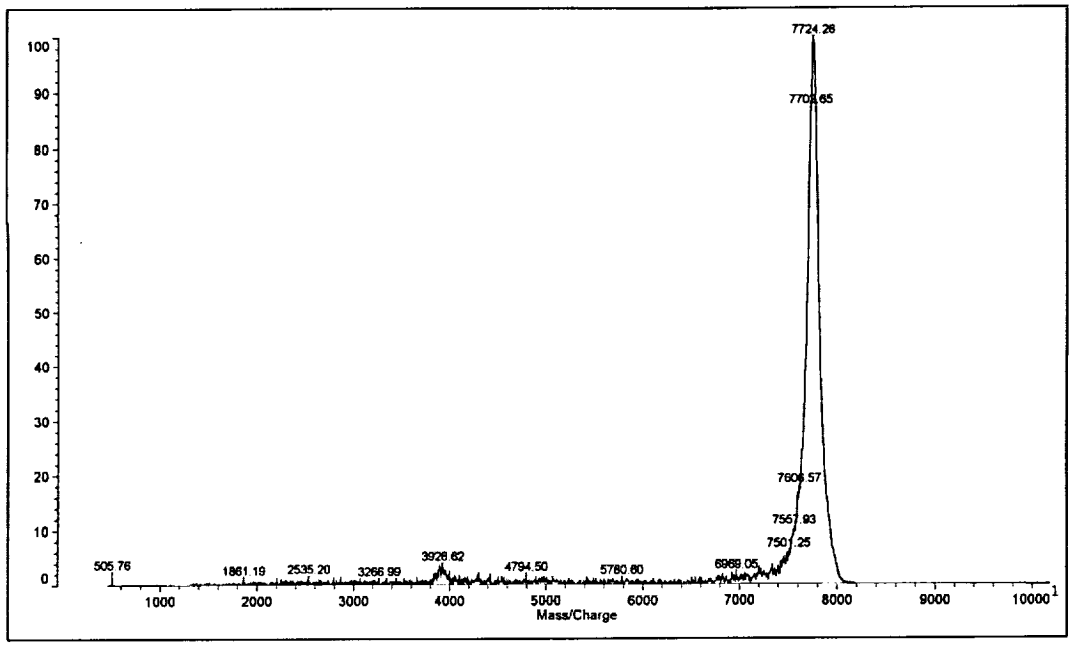


Figure 8. MALDI mass spectrum of insulin-like growth factors (IGF-I) (1.2 pmoles).

Under appropriate conditions, myofibrillar protein degradation can be estimated by determining urinary 3-methylhistidine (3MH) excretion rates. 3MH is an analog of histidine found predominantly in muscle actin and myosin. 3MH cannot be re-utilized and is rapidly and quantitatively excreted in the urine. TOFMS is being studied as a sensitive and rapid method for detection of 3MH in urine to measure muscle atrophy. The structure of 3-methylhistidine is shown in Chemical Structure II.

Figure 9 is a MALDI mass spectrum of 3-methylhistidine (concentration: 500 fmole).

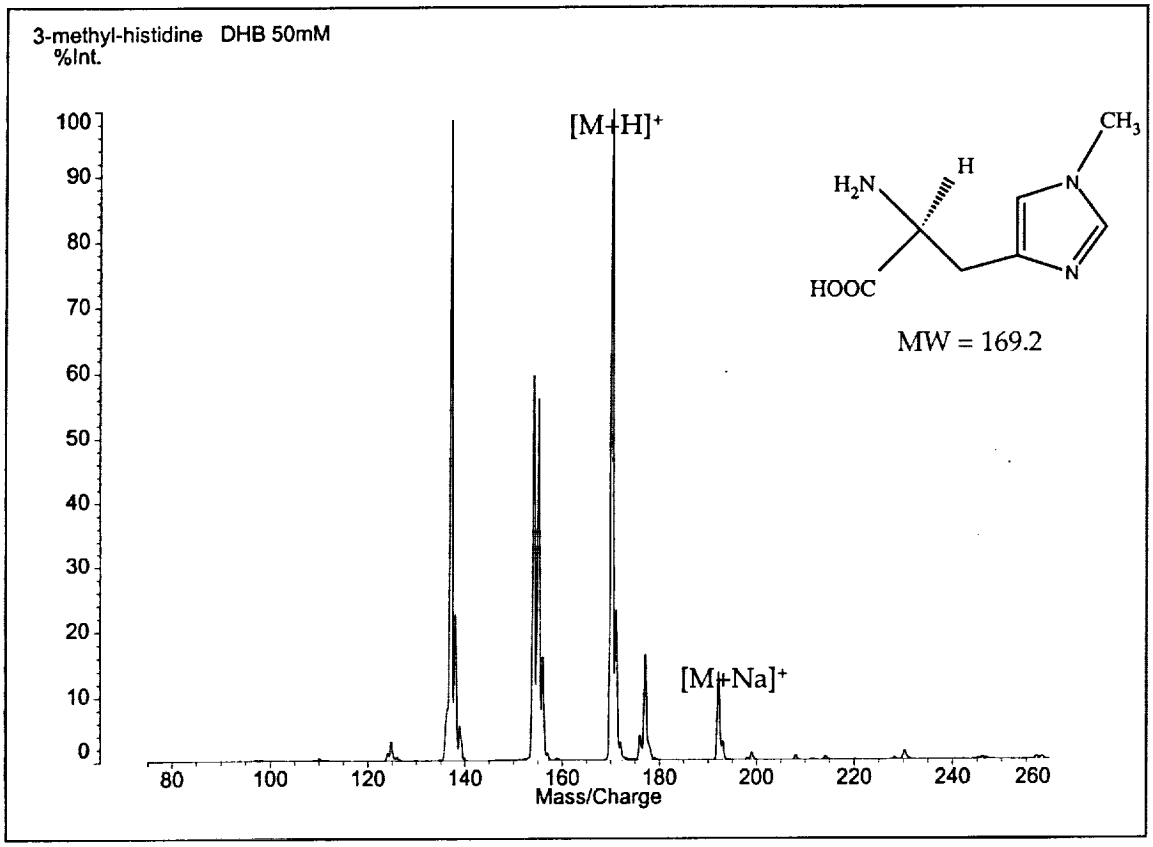
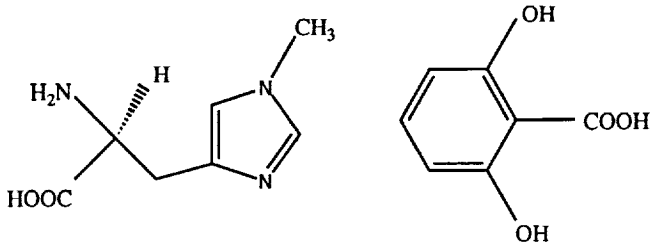


Figure 9. MALDI mass spectrum of 3-methylhistidine (concentration: 500 fmole).

Ions observed represent the protonated molecular ion for 3-methylhistidine, $[M+H]^+$, as well as its sodium adduct, $[M+Na]^+$. Other ions present in the spectrum are from 2,5-dihydroxybenzoic acid, the MALDI matrix.



Chemical Structures II: 3-Methylhistidine and 2,5-Dihydroxybenzoic Acid

Figure 10 is the MALDI mass spectrum of 2,5-dihydroxybenzoic acid (DHB).

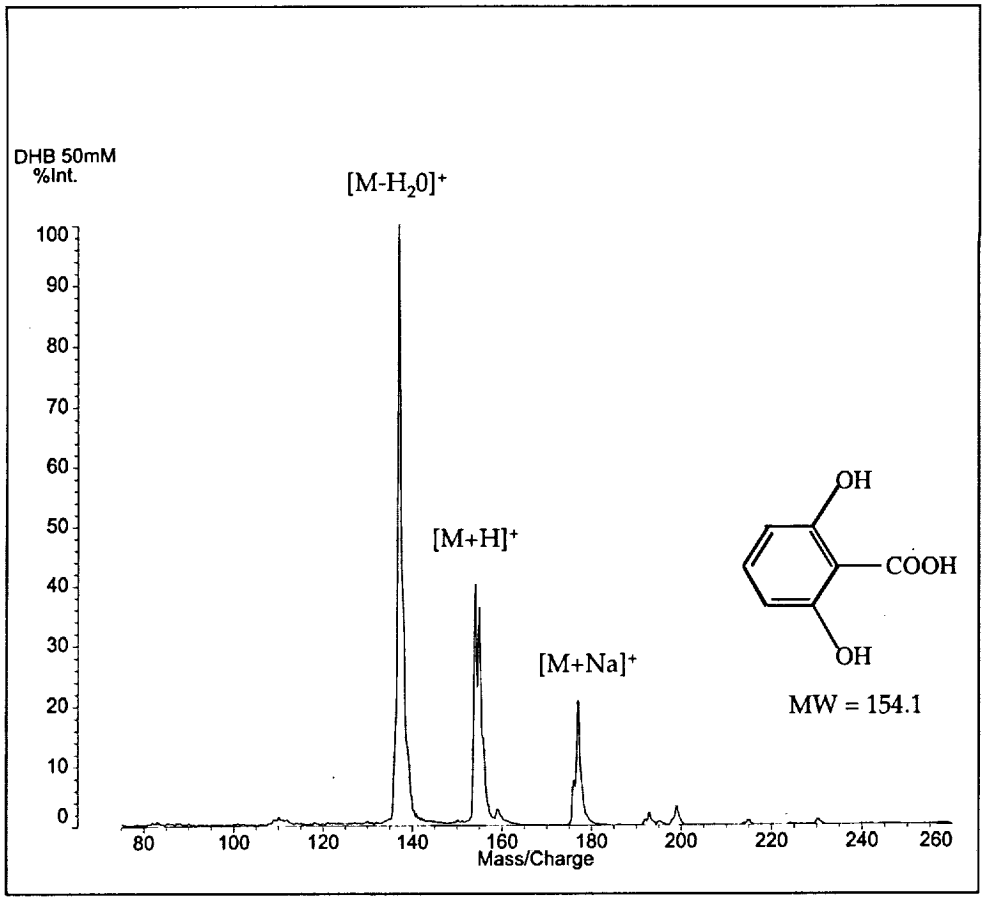


Figure 10. MALDI mass spectrum of 2,5-dihydroxybenzoic acid (DHB).

The molecular weight of this compound is 154.1 g/mole. This compound is the MALDI matrix. It shows peaks corresponding to the protonated molecular ions, $[M+H]^+$ and $[M-H_2O]^+$. Also present is the molecular ion $[M+Na]^+$. The structure of 2,5-dihydroxybenzoic acid is shown in Chemical Structure II.

Figure 11 displays the MALDI mass spectrum of human urine in the mass/charge range that the above biomarkers are present. The sample was not subjected to pretreatment or filtration. Molecular ions observed are indicative of the various components present in human urine.

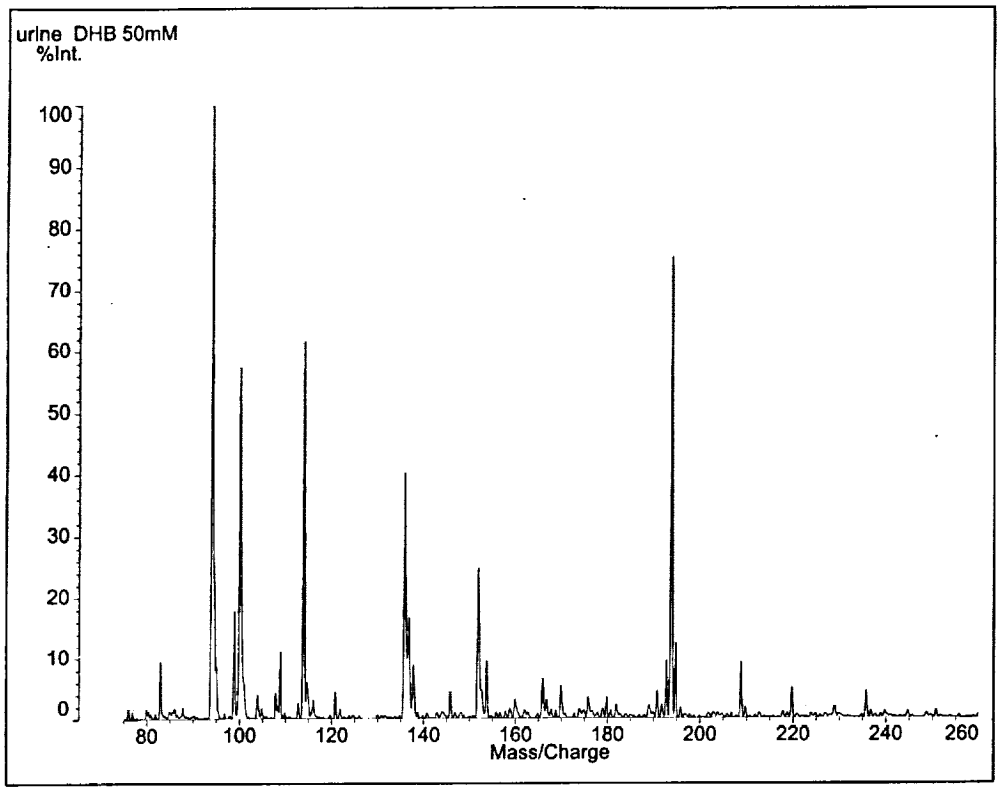


Figure 11. Time-of-Flight MS of Human Urine in 2,5-dihydroxybenzoic Acid Matrix (Kratos PC-Kompact MALDI 4; (+) Ionization Potential; Reflectron Power: 155)

Figure 12 is the MALDI mass spectrum of a human urine sample spiked with 3-methylhistidine (1ppm). Peaks corresponding to the $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ molecular ions of 3-methylhistidine are easily identifiable on this spectrum.

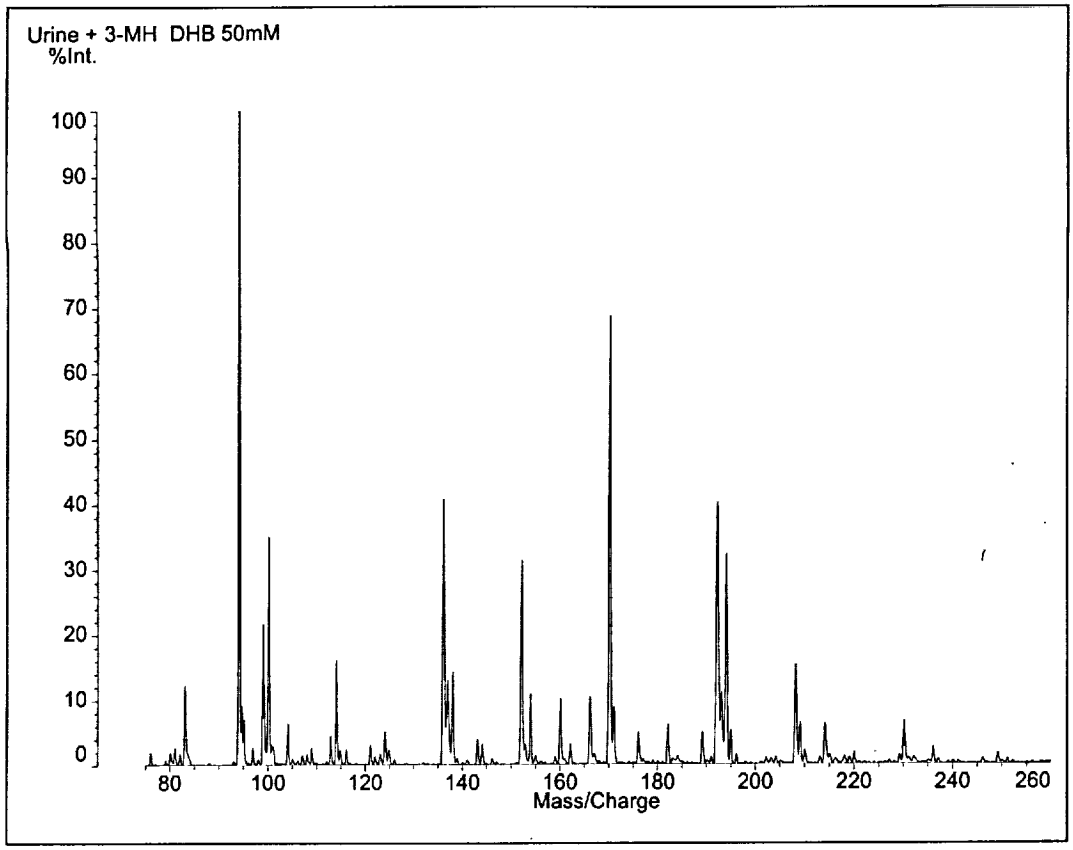


Figure 12. MALDI mass spectrum of a human urine sample spiked with 3-methylhistidine (1ppm).

Other peaks present in the spectrum are representative of components present in human urine (see Figure 11) and 3-methylhistidine without DHB matrix (see Figure 9).

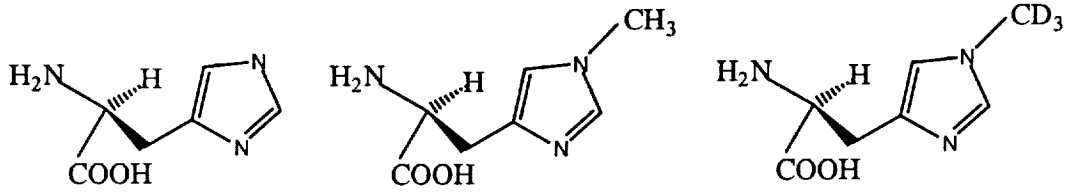
C. Quantitative Determination of 3-Methylhisitidine in Urine by Matrix-Assisted Laser Desorption Mass Spectrometry

3-Methylhistidine (3-MH) is a breakdown product of muscle protein and is quantitatively excreted in urine. 3-MH has been identified by several research teams as a biomarker for muscle atrophy. We have begun a study to investigate the use of matrix-assisted laser desorption mass spectrometry as a tool to quantitatively measure 3-MH in biological fluids.

First several biological biomarkers identified by the muscle atrophy and bone demineralization teams were analyzed in water and in urine to determine if MALDI was a feasible method for their analysis. All compounds investigated could easily be identified in the resulting MALDI spectra by the presence of strong molecular ions representative of each biomarker. Additionally, we analyzed several MALDI matrices to determine which was most applicable for the widest range of biomarkers. 2,5-Dihydroxybenzoic acid (HDB) was chosen since this matrix gave analytical data for a large range of compounds with the least amount of spectral overlap between matrix and analyte ions. This matrix was also found to give more homogeneous crystal structures yielding strong molecular ion signals and good shot-to-shot reproducibility.

After successful analysis of pure biomarkers in water and urine, efforts were focused on quantitatively measuring these compounds in biological fluids. We began by analyzing various concentrations of 3-methylhistidine in water and in urine to determine a rough estimate of the relationship between analyte concentration and analyte molecular ion intensity. The concentrations used in this study were based on 3-methylhistidine concentration typically found in urine, i.e. 20pmole – 3.5nmole. Standard curves were prepared to analyze the data.

In a second series of experiments, internal standards were incorporated to accurately measure the relationship between analyte concentration and ion intensity. Internal standards compensate for deviations associated with sample preparation, detector response, and data acquisition. We have examined the utility of two types of internal standards, histidine, a structural analogue, and d_3-3-methylhistidine, a stable-isotope labeled analogue. The chemical structures are shown below:



Histidine 3-Methylhistidine d_3-3-Methylhistidine

Chemical structures III.

3-Methylhistidine (3-MH) samples in water and urine were prepared ranging from 5 uM – 10mM, keeping the (3MH)/(histidine) ratio constant at 1:10. Protonated

molecular ions for 3MH and histidine could be identified in the corresponding MALDI spectra.

Figure 13 displays a spectrum of a urine solution containing elevated amounts of 3MH using histidine as the internal standard. Molecular ions present include the protonated molecular ion for 3-MH at m/z 170.6 and the protonated molecular ion for histidine at m/z 156.5. Quantitative data could be obtained after analysis of all spectra, however the accuracy of this method was questionable due to spectral overlap of the protonated histidine molecular ion with an isotope peak of DHB, the MALDI matrix. For these experiments, samples were prepared by mixing a known aliquot of 3-MH spiked urine with a histidine solution and depositing 0.5uL on a MALDI sample slide. DHB (0.5uL, 50mM in ACN/0.1% TFA/H_2O 70:30, v/v) was then added. The sample/matrix mixture was allowed to air-dry before insertion into the mass spectrometer for analysis. Mass spectrometry was performed on a Kratos Kompact MALDI-4 time-of-flight mass spectrometer equipped with 337nm nitrogen laser operating in the positive-ion and reflectron modes of detection.

To overcome the problem of spectral overlap between histidine and DHB, the use of d_3-3-methylhistidine (d_3-3-MH) as an internal standard was examined. Use of a stable-isotope labeled internal standard has several advantages. These include similar extraction efficiencies between the analyte and standard, allowing more accurate quantitative comparison since there is minimal change in the chemical properties of the two substances, but a distinct physical change easily characterized by mass analysis. MALDI spectra generated using d_3-3-MH as the internal standard are shown in Figures 14-16. The spectrum, Figure 14, is from a standard urine solution containing 300uM of d_3-3-MH.

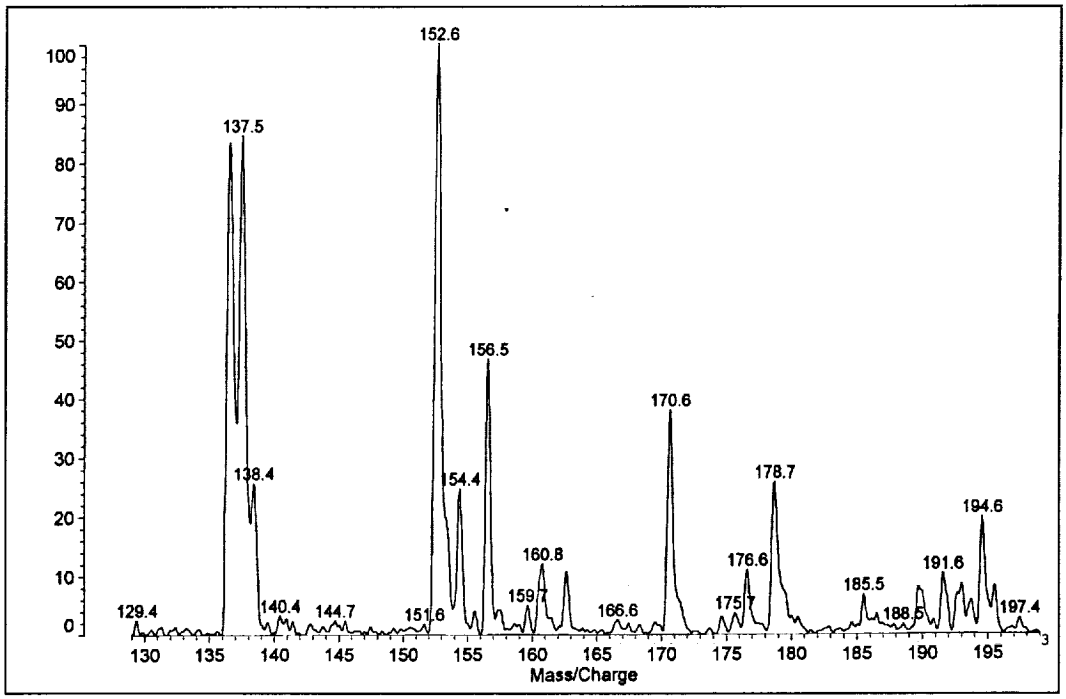


Figure 13. Urine containing elevated amounts of 3MH using histidine as an internal standard.

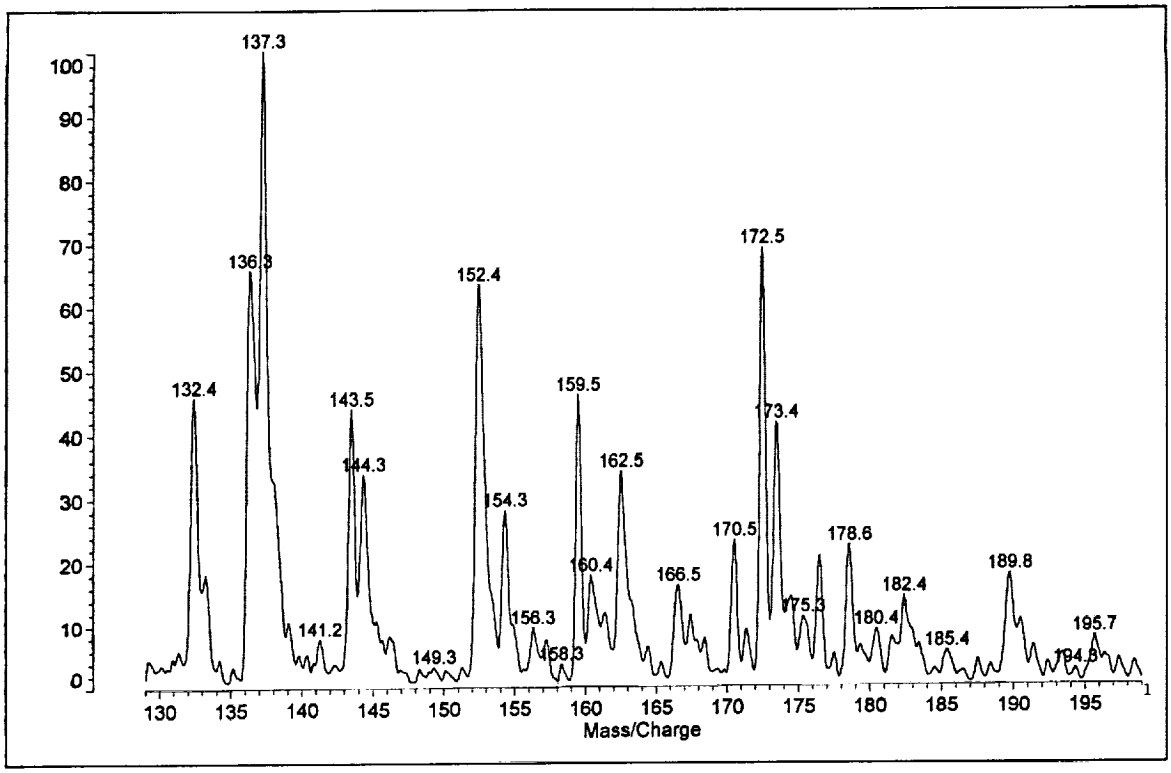


Figure 14. Standard urine solution containing 300 uM of d_3-3-MH.

Although this is a relatively high concentration of internal standard, due to the complexity of urine solutions a high concentration was need to overcome signal suppression effects. The protonated molecular ion for d_3-3-MH is observed at m/z 173.4 and is easily resolved from other ions present in the spectrum. Also, analysis of the DHB matrix did not reveal any ions in this region, thereby preventing any problems associated with spectral overlap. The spectrum of a urine solution spiked with 3-MH and containing the d_3-3-MH internal standard is shown in Figure 15.

Protonated molecular ions are easily identifiable for both substances at m/z 170.9 and 173.8 respectively. Figure 16 is a representative spectrum of a standard aqueous solution of 3-MH and d_3-3-MH.

Protonated molecular ions for both compounds are observed at m/z 170.7 and 173.8. Remaining ions in the spectrum are from the DHB matrix. All spectra were generated by signal averaging 50-laser shots rastered over a 1mm sample well into a single spectrum. Typically, 0.5uL of sample solution (3-MH concentration range analyzed was 1.56 pmole – 5 nmole), is applied to the well followed by 0.5uL of 50mM DHB matrix (in ACN/0.1%TFA/H2O 70:30, v/v). (The sample/matrix mixture is allowed to dry in ambient air prior to insertion into the mass spectrometer for analysis. A plot of the ratio of relative peak intensities of (3MH)/(d_3-3-MH) versus 3-MH concentration gave a linear response with a correlation coefficient, $R^2 = 0.9799$ and a relative standard deviation of the slope of 4.00% (Figure 17).

A standard curve in an aqueous solution also gave a linear response with a correlation coefficient, $R^2 = 0.9896$, and a relative standard deviation of the slope of 3.18% (Figure 18). Each data point is the average of three 50-laser shot spectra taken for each sample.

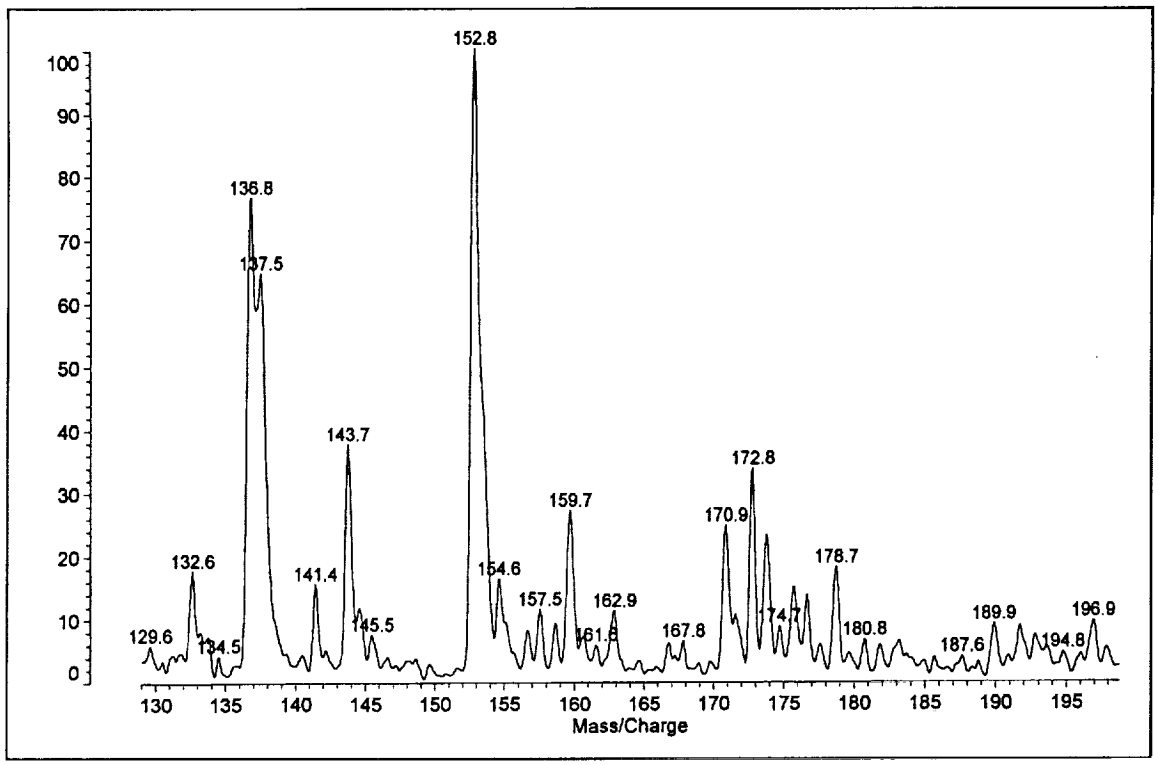


Figure 15. Spectrum of urine solution spiked with 3-MH and d_3-3-MH internal standard.

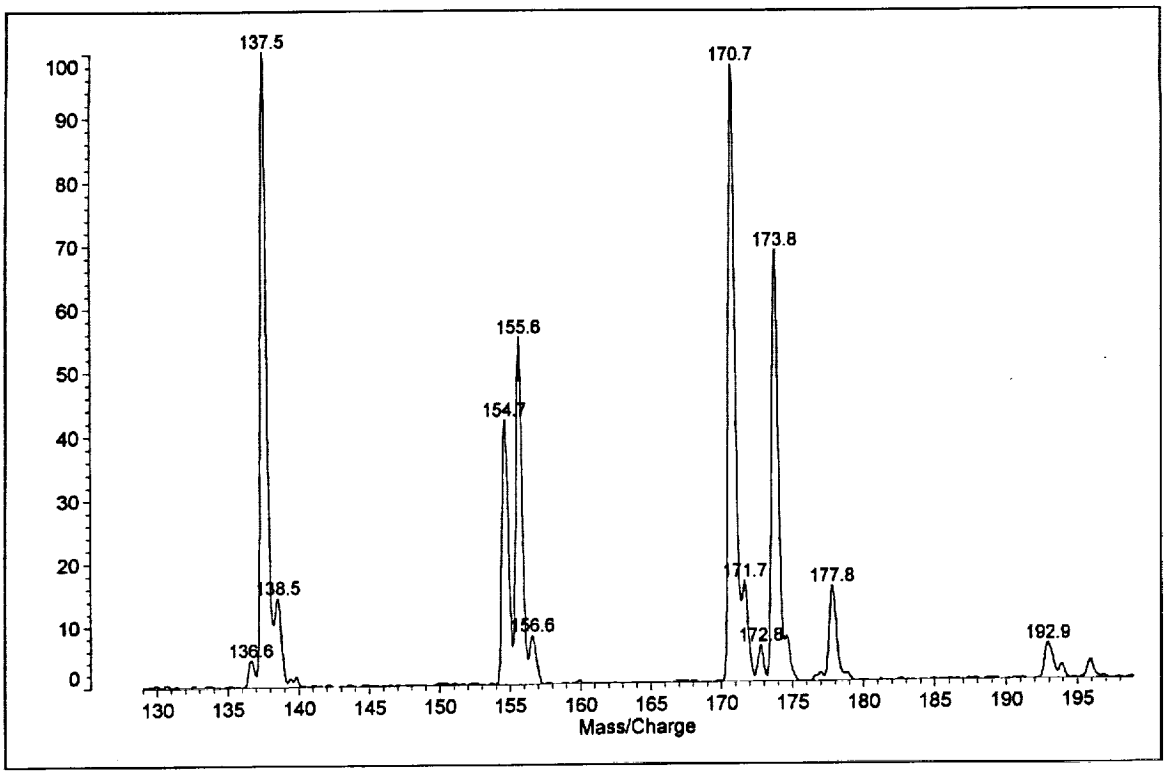


Figure 16 is a representative spectrum of a standard aqueous solution of 3-MH and d_3-3-MH.

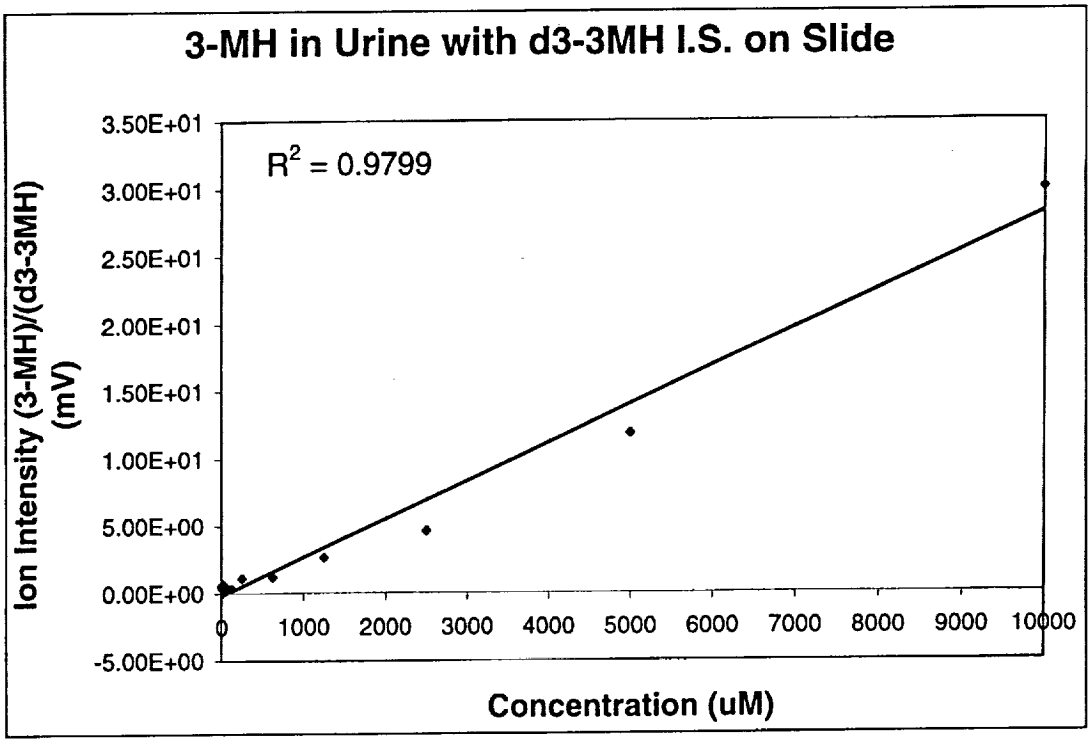


Figure 17. Plot of the ratio of relative peak intensities of (3MH)/(d_3-3-MH) versus 3MH.

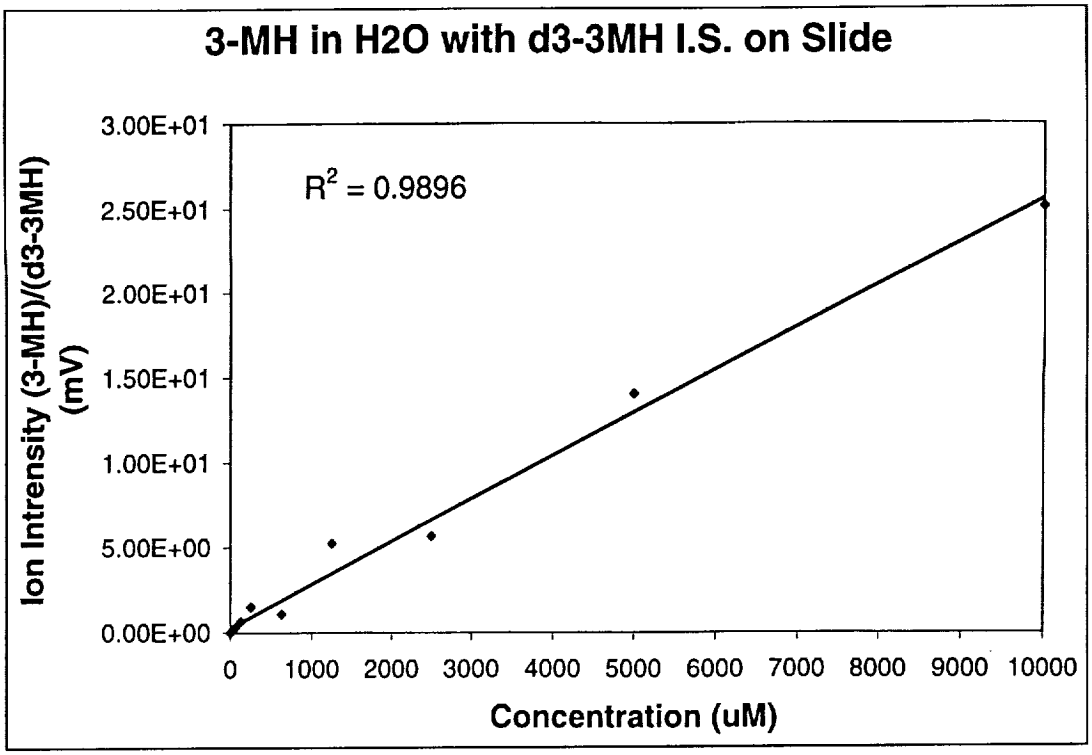


Figure 18. A standard curve in an aqueous solution also gave a linear response with a correlation coefficient, R^2 = 0.9896 and a relative standard deviation of the slope of 3.18%. Each data point is the average of three 50-laser shot spectra taken for each sample.

Manual deposition of sample and matrix proves difficult in obtaining uniform crystallization of the sample/matrix mixture. One way to compensate for this problem is to locate the "sweet" spot, i.e. the region on the MALDI slide that gives maximum ion signal for the analytes of interest; however, this presents more of a problem for accurate quantitation. We have addressed this issue by using internal standards and by investigating different surface preparations such as electrospray and pre-coated slides as MALDI templates.

Electrospray Apparatus

Quantitative electrospray sample deposition has been demonstrated by Professor Kevin G. Owens at Drexel University to significantly aid in the quantitative analysis of biological molecules by minimizing spot-to-spot and shot-to-shot variations. He recently published reproducible analysis of biomarkers to 1% coefficient of variation for MALDI spectra.[910] We built and used an electrospray sample deposition device to prepare MALDI samples according to a recently reported design.

We used the electrospray sample deposition method to deposit uniformly thin nitrocellulose films. Nitrocellulose has been shown to increases the [M + H*] ion yield by reducing the level of impurities. The nitro groups in nitrocellulose electrostatically bind the analyte preferentially over impurities. This allows impurities to be rinsed away without depleting the analyte. Nitrocellulose has also been shown to improve the sample-to-sample reproducibility of MALDI ion yield because it modifies the

crystallization of matrix/analyte solution to allow even coverage over the sample surface.

The apparatus shown in figure 19 consists of a HV power supply, an infusion pump (syringe pumping system with microliter/minute flow rates), and a solvent reservoir. Fluidics were controlled manually by three 3-way valves, a small-bore stainless steel capillary electrospray needle and adjustable sample stage that was electrically grounded.

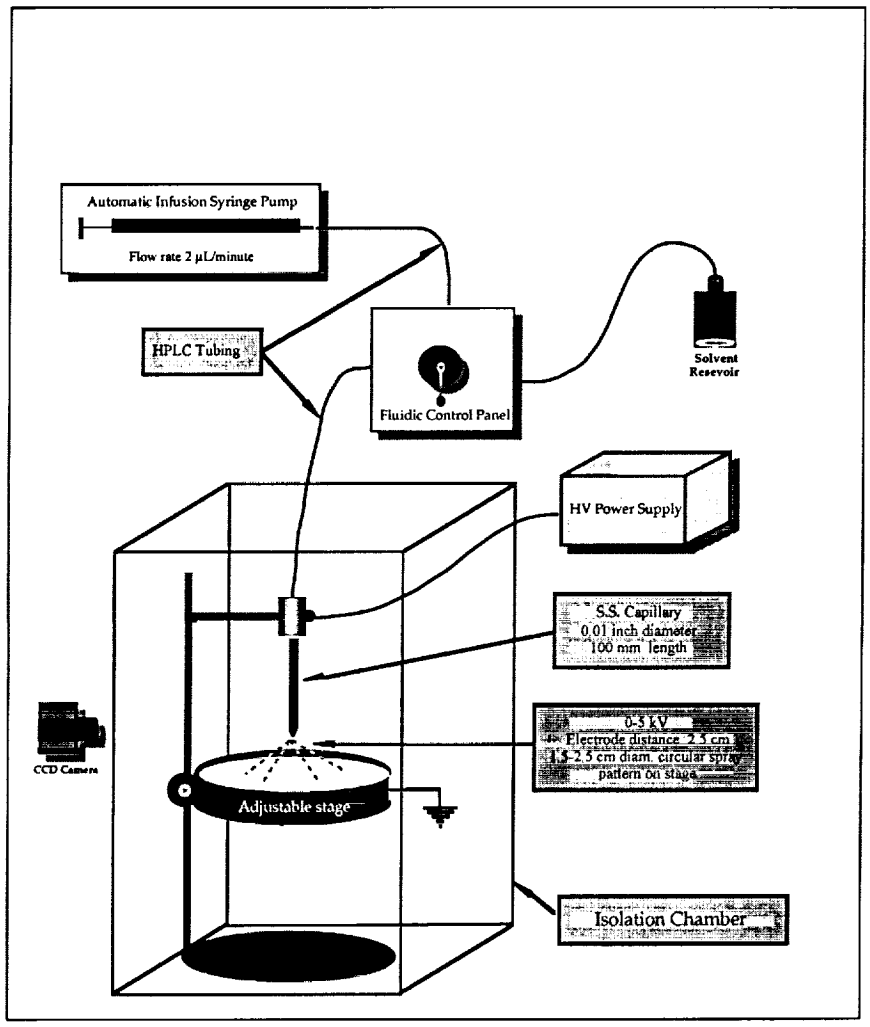


Figure 19. Schematic diagram of electrospray apparatus.

Nitrocellulose (Aldrich #9004-70-0) was deposited at a constant rate from the methanol solution. The mixture is sprayed directly onto a Kratos commercial sample slide that was in electrical contact to the sample stage. Electrospray deposition was accomplished by introducing approximately 5-10 microliters of sample mixture into the system. A potential of approximately +4.75 kV was applied to the capillary while the sample probe tip is held at ground a distance of 20 mm away. A flow rate of approximately 10-20 microliter per minute is applied using the infusion pump. With this set of conditions, a circular spray pattern was observed to build up on the sample stag

covering an area of approximately 2-2.5 cm in diameter. The distance between the capillary tip and grounded probe and the applied voltage was maintained to obtain a stable, elongated Taylor cone. The flow rate was adjusted according to the solution conditions to avoid sputtering of the polymer onto the sample stage.

Solutions of nitrocellulose + matrix (α-cyano-4-hydroxycinnamic acid) (α-CHCA) were also successfully sprayed directly on to Kratos commercial sample slides using a similar set of deposition conditions

MALDI time-of-flight mass spectrometry was performed on the nitrocellulose films and on the nitrocellulose / matrix thin films. The spectra showed that the electrospray deposition was an extremely effective method to prepare thin polymer films, and the method could also be used to uniformly pre-deposit matrix onto sample stages. Nitrocellulose / matrix thin films were then spotted with neurotensin. Neurotensin is a regulatory hormone; it was used in these studies as a standard to evaluate the ability of the nitrocelloluse strengthen the [M + H*] ion yield. The spectra revealed a single strong peak was observed at 1665.5 Da which corresponds to is the [M + H*] ion for neurotensin. These results demonstrate that electrospray deposition is an important method to preprocess samples prior to mass spectral analysis.

Pre-Coated MALDI Slides

We have also investigated the use of MALDI slides pre-coated with DHB matrix to address this problem of inhomogeneous sample/matrix crystallization. An even layer of MALDI matrix results in a fairly uniform distribution of sample across the sample spot and less variability in sample ion production. The results from these initial studies appear promising. We obtained a more linear relationship between molecular ion signal and concentration of 3-MH, using histidine as the internal standard, when the sample was applied onto a pre-deposited DHB matrix foil compared to manual deposition. These experiments were also conducted using the internal standard d_3-3MH. Molecular ion signals can be observed for all components of interest, at m/z 170.4 $[3\text{-MH} + H]^+$ and m/z 173.6 $[d_3\text{-3-MH} + H]^+$. Additional work is ongoing to determine the best concentrations of matrix to lessen the overpowering effect of matrix ions relative to analyte ions (Figure 20).

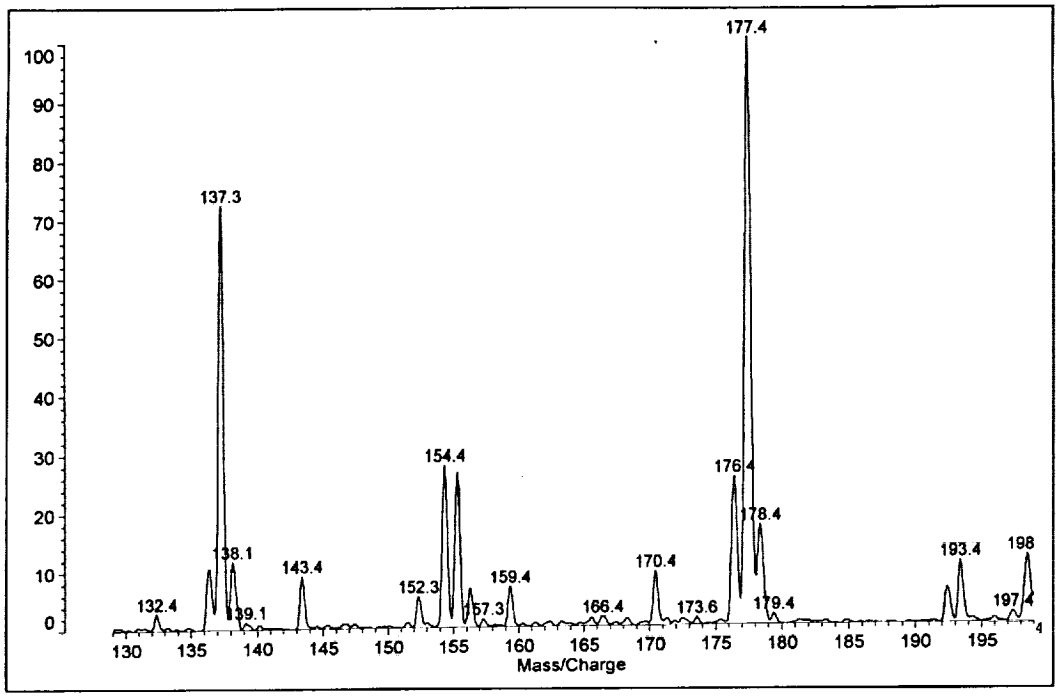


Figure 20. Spectrum of near linear relationship between molecular ion signal and concentration of 3-MH, using histidine as the internal standard.

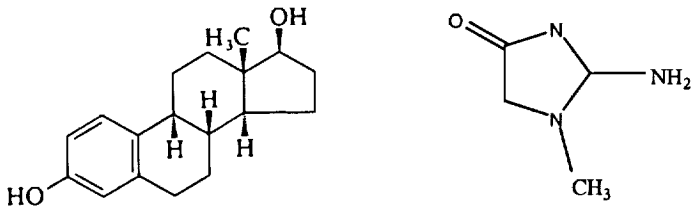
D. Collaboration with Professor Jay R. Shapiro at JHU/SOM to Integrate TOFMS Development into Bone Demineralization / Calcium Metabolism Project

The aim of this research is to validate that the miniature time-of-flight mass spectrometer is an important diagnostic tool that can be applied to measure important bone biomarkers and the effectiveness of applied countermeasures in human urine samples. We investigated metabolic bone markers hydroxyproline, pyridinium crosslinks, and deoxypyridinoline crosslinks.

Exposure to microgravity rapidly leads to osteopenia due to increased bone resorption and decreased bone formation. Studies with Skylab and Russian crews demonstrated 1.0-1.6%/month mean losses of bone mass from the spine, femur, neck, and pelvis increasing the risk of fracture. In addition, the mobilization of calcium from bone, as much as 300 mg/day, leads to an increased risk of nephrolithiasis. Alterations in skeletal metabolism pose substantial risks as mission duration is extended. Also of concern is the lack of evidence that bone loss is reversible on return to earth. Progress in developing effective countermeasures to demineralization depends on increased understanding of how the complex biochemical systems that modulate bone turnover response to pharmacological and stress-induced interventions.

The Bone Demineralization/Calcium Metabolism Project has established a need for identifying levels of several biological markers, including growth hormone secretion (IGF-I), trivalent hydroxypyridinium crosslinks, creatinine, vitamin D, serum and urine calcium, glucocorticoids (stress function and catabolic factor), and parathyroid hormone (PTH). The development of effective countermeasures will depend on increased understanding of biochemical systems that modulate bone turnover response to pharmacological / stress-induced interventions.

The JHU/APL team has demonstrated results that show the technical feasibility for evaluating bone demineralization/calcium metabolism biomarkers by TOFMS at physiological concentrations. Figures (21 - 24) give examples of spectra obtained on the MALDI-TOF test bed.



Estradiol
[1,3,5(10)-estra-triene-3,17β-diol]

FW = 113.1

Chemical Structures IV. Estradiol and Creatinine

Figure 21 shows the MALDI mass spectrum of human urine spiked with creatinine. Upon comparison with the mass spectrum of unspiked human urine in Figure 11, peaks representative of creatinine are observed at m/z 114.0, 135.9, and 152.0 Da. These correspond to the $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ creatinine molecular ions,

respectively, and are in excellent agreement with the calculated values of 114, 136, and 152 Da, respectively. The structure of creatinine is shown in Chemical Structure IV.

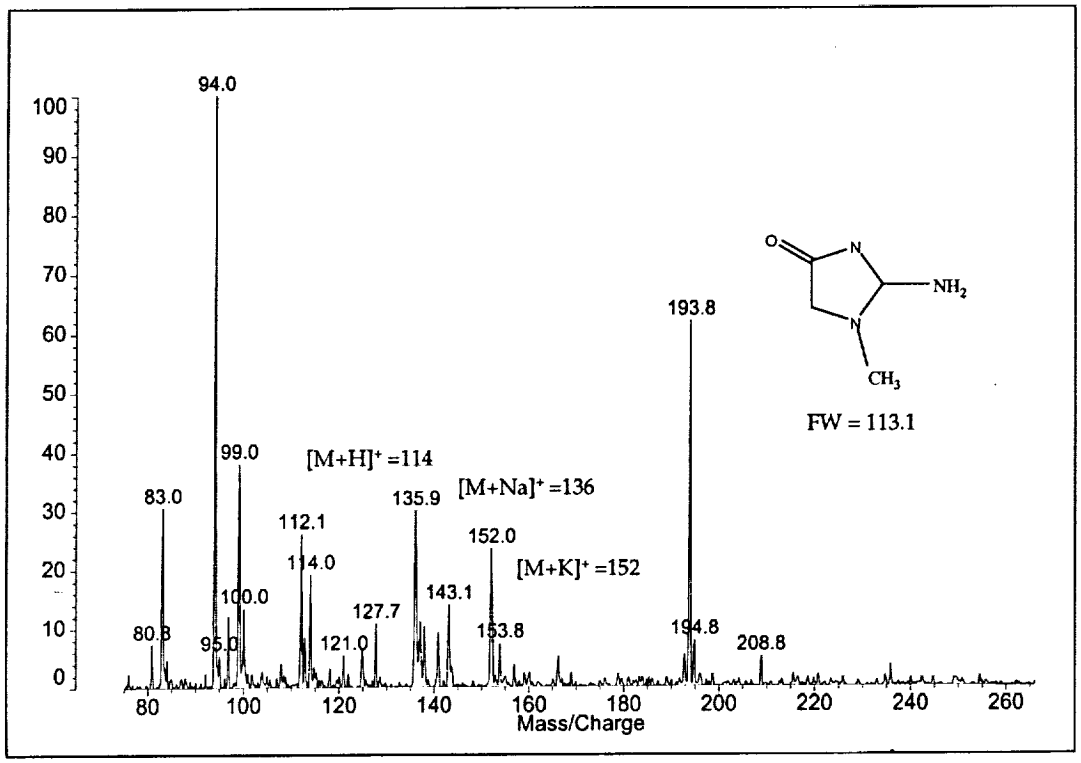


Figure 21. Time-of-Flight MS of Creatinine in Urine in DHB ($1X10^{-4}$ M)
(Daily Output: 25 mg per kg of body weight per day)
(Kratos PC-Kompact MALDI 4; (+) Ionization Potential; Reflectron Power: 150)

In bone and cartilage, collagen is bound by pyridinoline (pyr) or hydroxypyridinoline (deoxypyridinoline, (D-pyr)) crosslinks. D-pyr is found exclusively in bone, while pyr is found in skin, joint and cartilage. The ratio of pyr/D-pyr is approximately 2:3.[11] Pyridinoline crosslinks are released into the circulation during bone resorption and are excreted as both free pyridinolines (deoxypyridinoline and pyridinoline). There are several different ways to measure pyridinoline crosslinks. The most sensitive and accurate methodology for urinary pyridinoline crosslinks excretion is high performance liquid chromatography (HPLC), which permits quantitation of total crosslinks into D-pyr and pyr.[12] The disadvantages of HPLC are cost, clinical utility of HPLC, and capability of technicians to perform laborious multi-step laboratory protocols. Direct measurement of these crosslinks by TOFMS will provide a powerful tool to help determine bone loss in space.

Figure 22 shows the MALDI mass spectra of a pyridinoline / deoxypyridinoline (Pyd/Dpd) standard solution (back spectrum) and DHB (front spectrum). Protonated molecular ions for Pyd and Dpd are observed at 412.9 and 428.9 Da in addition to matrix adducts. The values obtained experimentally are in close agreement with the calculated molecular weights of 413 and 429 Da, respectively. The structure of deoxypyridinoline and pyridinoline are shown in Chemical Structure V.

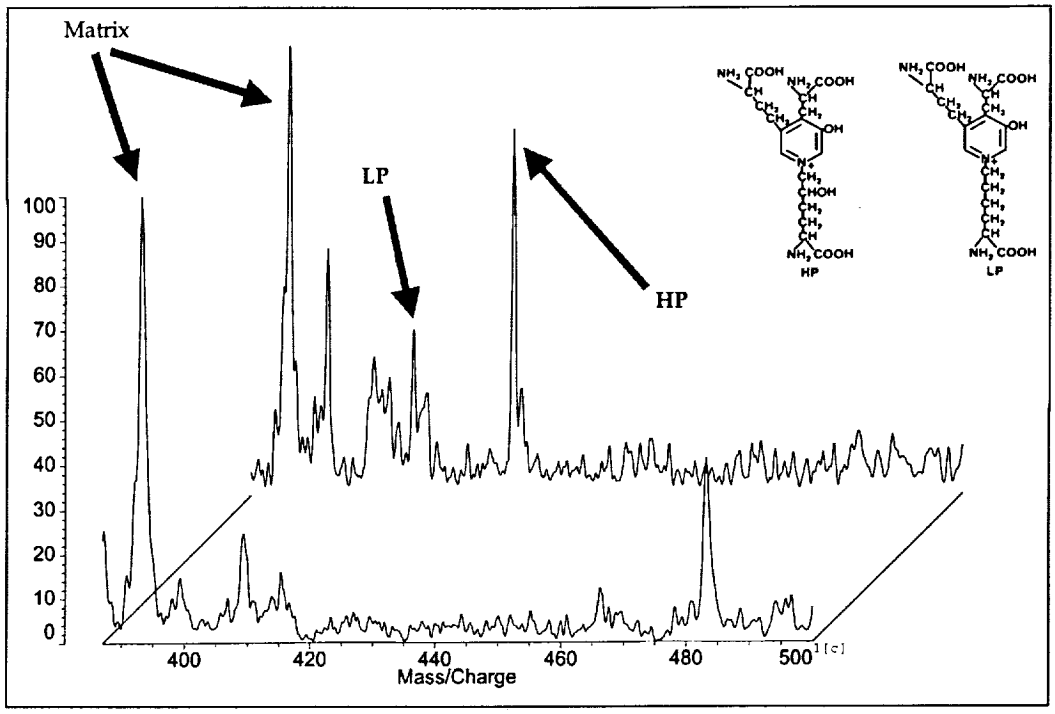
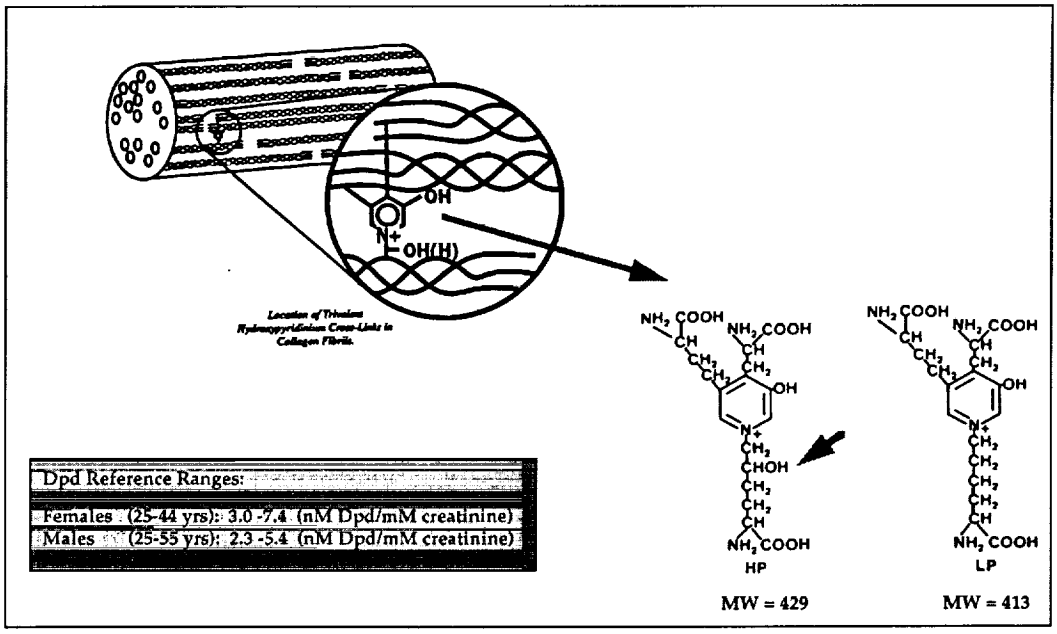


Figure 22. Time-of-Flight MS of Trivalent Hydroxypyridinium Crosslinks (Kratos PC-Kompact MALDI 4; (+) Ionization Potential; Reflectron Power: 100; 100% = 18mV)

There is a critical interplay between mechanical and hormonal factors in the maintenance of muscle mass and bone density when challenged by a state of microgravity. It is widely speculated that steroid hormones play a central role in the early stages of muscle atrophy and bone demineralization.



Chemical Structures V. Structures of Trivalent Hydroxypyridinium Crosslinks Anchored to Type I Collagen Peptides in Bone Matrix.

Figures 23 and 24. The MALDI mass spectrum of estradiol (Figure 23) is very similar to the mass spectrum of DHB, the MALDI matrix. See Figure 24.

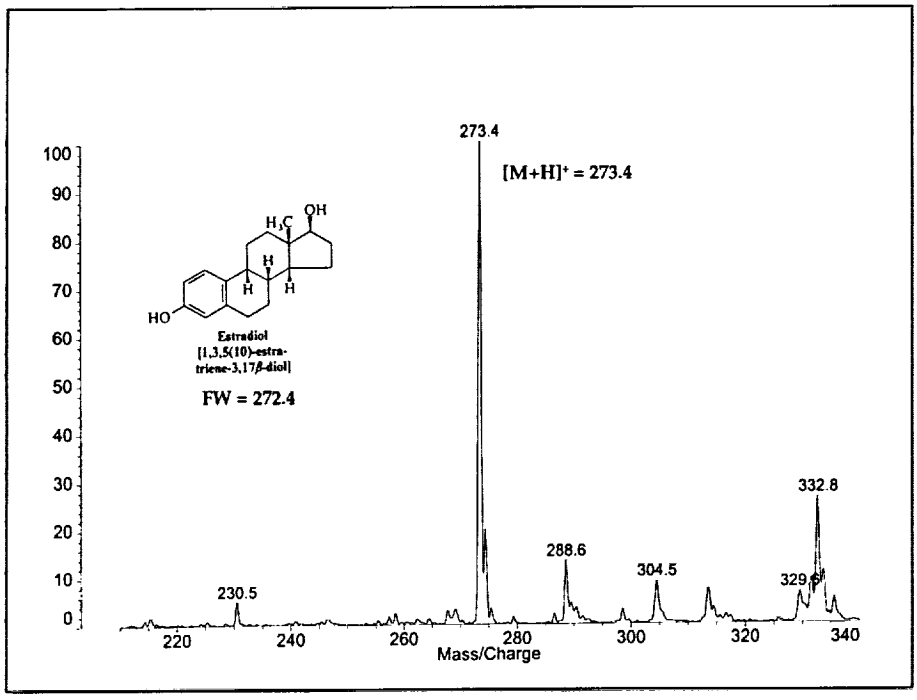


Figure 23. Time-of-Flight MS of Estradiol in DHB (1micromole)
Kratos PC-Kompact MALDI 4; (+) Ionization Potential; Reflectron Power: 95; 100% = 85mV

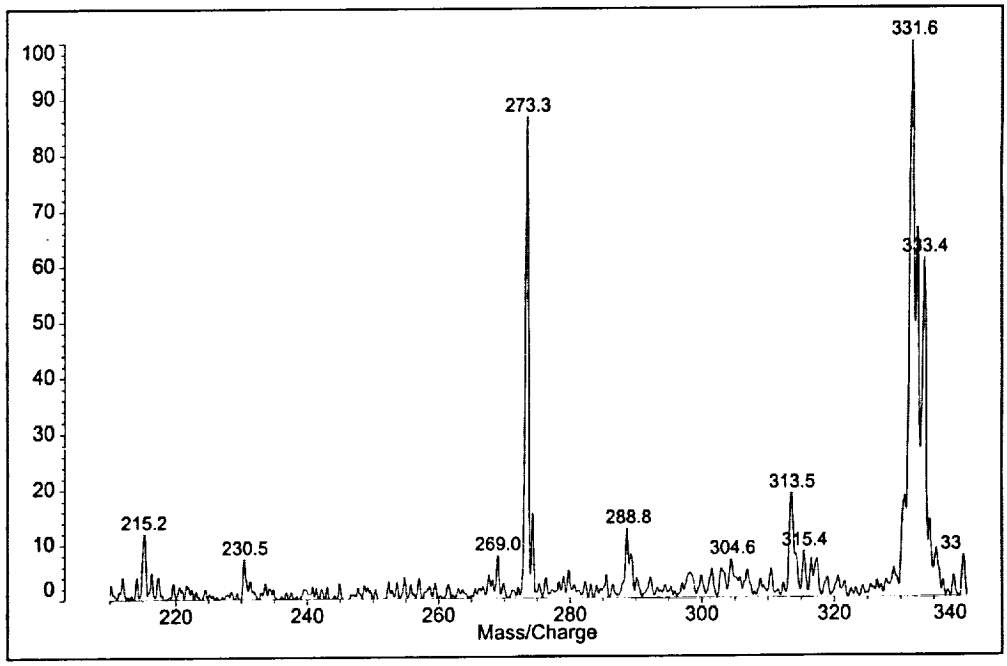


Figure 24. Reflectron Time-of-Flight MS of DHB Matrix Kratos PC-Kompact MALDI 4; (+) Ionization Potential; Reflectron Power: 95; 100% = 5mV.

However, upon close examination, the peak appearing at m/z 273.4 in Figure 23 is of much higher intensity than the corresponding ion at m/z 273.3 in Figure 24. This dramatic increase in ion abundance is due to protonated estradiol, which has a calculated molecular weight of 273.4 Da. The peak appearing at (m/z 273.3 Da) in the DHB spectrum corresponds to the protonated DHB-H_2O dimer, this has a calculated molecular weight of 273.2 Da. The structure of estradiol is shown in Chemical Structure IV.

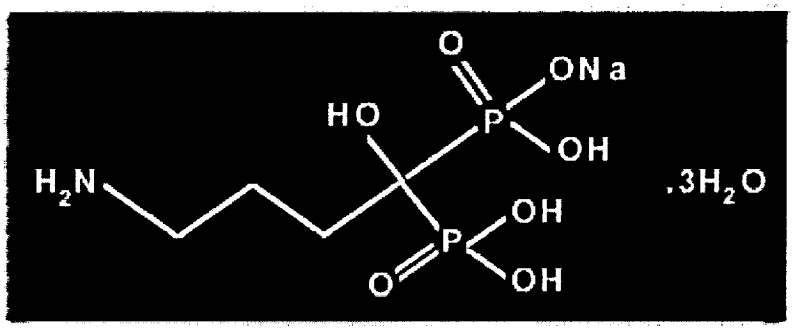
Experimental

MALDI MS analysis was conducted on a Kratos Kompact MALDI IV (Shimadzu, Columbia, MD) mass spectrometer equipped with a 337nm-nitrogen laser. The Kratos Kompact MALDI IV is similar in design to the JHU/APL miniature MS. The Kratos Kompact MALDI IV uses key design features provided by Professor Cotter. Professor Cotter has made major contributions to the design of the TOFMS for this project. Data was acquired in reflectron mode and the matrix used was 2,5-dihydroxybenzoic acid (50mM in 70:30 v/v ACN/0.1%TFA/H_2O). Samples were analyzed by placing 0.5uL of sample on a MALDI slide followed by 0.5uL of matrix. The spot was allowed to dry in ambient air and inserted into the mass spectrometer for analysis.

Bisphosphonate: An Effective Countermeasure to Prevent Bone Loss

Muscle atrophy and bone losses are major obstacles to extended spaceflight and extraterrestrial habitation. Chronic immobilization or weightlessness decrease muscle strength and increase the risks of fracture. This is common with spinal cord injury patients, in the elderly and in astronauts exposed to microgravity. Jay Shapiro, M.D. is conducting a research program at the National Rehabilitation Hospital that addresses the problem of bone loss following spinal cord injury (SCI) in tetraplegic and paraplegic subjects. He is utilizing SCI subjects as a model for the muscle and bone loss experienced during extended spaceflight.

Bisphosphonate administration to the hindlimb of suspended rats and limb immobilization studies in dogs suggest that this compound is an effective countermeasure to bone loss.[13] Alendronate is a member of the bisphosphonate family of drugs used to treat/prevent osteoporosis. The structure of alendronate is shown below.



Chemical Structure VI. Fosamax (Alendronate).

Clinical studies have been performed to investigate the pharmacokinetics and

pharmacodynamics of alendronate. Alendronate is one of the most potent bisphosphonates currently undergoing clinical investigation (>100-fold more potent than etidronate *in vivo*). Alendoronate shows an absence of systemic toxicity. The pharmacokinetics of alendronate are similar to those of other bisphosphonates. It is also used to treat some bone diseases and some cases of cancer that have spread to bones. Recently an FDA advisory committee recommended the approval of alendronate (Fosamax/Merck), an aminobisphosphonate that has proved to be well tolerated and highly effective for inhibiting osteoclastic bone resorption without interfering with bone formation. Merck is studying alendronate for both postmenopausal osteoporosis and Paget's disease. Alendronate is also suitable for administration to astronauts on extended flight in microgravity conditions such as the Mars flight/habitation estimated to last over 3 years.

Our long-term goal is to evaluate alandronate as a potential countermeasure for bone loss. We plan to measure the retention of alendronate in subjects in serum and urine. The effectiveness of the countermeasure will be determined based on its relationship to bone density and metabolism. Because the retention of alendronate doses > 50% based on urinary excretion, urine analysis should provide a useful method to monitor the effectiveness of this countermeasure. This countermeasure can also be assessed base on sustained inhibition of markers of bone resorption (N-TX), which we have also investigated.

We have performed a mass spectral analysis of alendronate to determine the mass spectral pattern by MALDI and to add the compound to our library of critical biomarkers. We determined the molecular weight of the compound from the commercially available product called Fosamax.

Fosamax (alendronate sodium) is a bisphosphonate that acts as a specific inhibitor of osteoclast-mediated bone resorption. Bisphosphonates are synthetic analogs of pyrophosphate that bind to the hydroxyapatite found in bone.

Alendronate sodium is (4-amino- 1 -hydroxybutylidene) bisphosphonic acid monosodium salt trihydrate. The empirical formula of alendronate sodium is $C_4H_{12}NNaO_7P_2 \cdot 3H_2O$ and its formula weight is 325.12. Alendronate sodium is a white, crystalline, non-hygroscopic powder. It is soluble in water, very slightly soluble in alcohol, and practically insoluble in chloroform. Fosamax is sold by the Merck Pharmaceutical Corporation. Fosamax tablets for oral administration contains alendronate monosodium salt trihydrate. Inactive ingredients include microcrystalline cellulose, anhydrous lactose, camauba wax, croscarmellose sodium, and magnesium stearate.

A single tablet was crushed into a powder and mixed with 3 ml of water. One ml of solution was filtered through 150-mm paper filter. The resulting liquid was mixed 50/50 with DHB matrix and deposited on the slide.

The following spectrum (figure 25) was obtained for Fosamax (bottom trace) compared to DHB matrix (top trace). The spectra shows a strong peak in the spectra for Fosamax at 365 which corresponds to the adduct of the compound with Ca^{2+} ions. (The molecular weight of Fosamax is 325.12.)

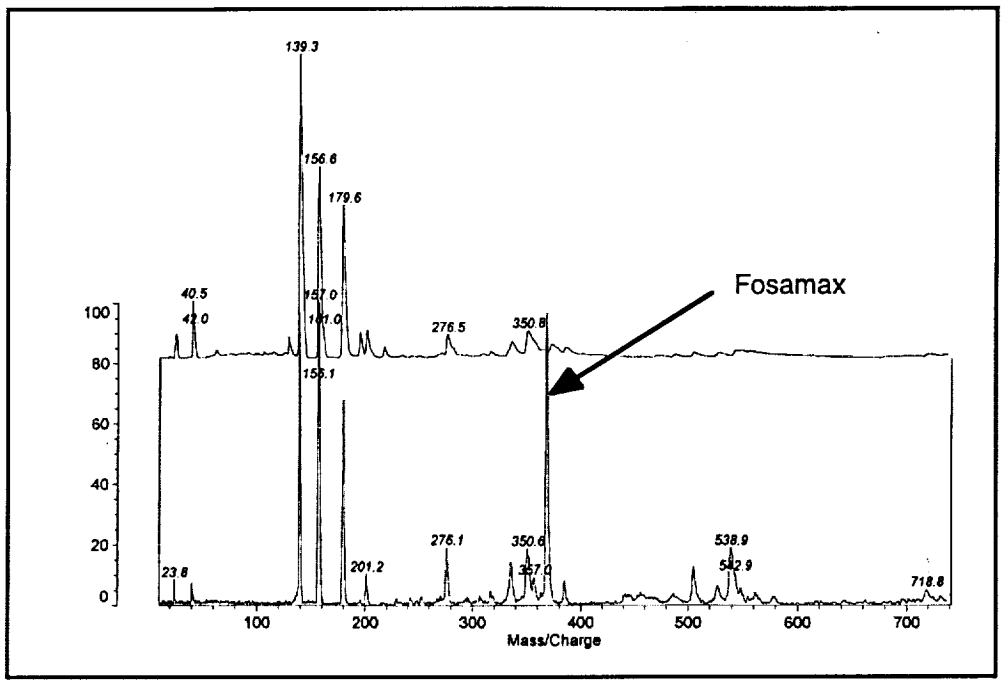


Figure 25. Fosamax (bottom trace) compared to DHB matrix (top trace). The spectra shows a strong peak for Fosamax at 365 m/e which corresponds to the adduct of the compound with Ca^{2+} ions.

E. Analysis of Biomarkers in Breath

The sampling of human breath in support of physiological investigation was addressed at the JHU School of Hygiene and Public Health (HPH).[1415] HPH investigators have detected breath ethane and pentane to monitor oxidative damage due to vitamin deficiency and total body irradiation.

Traditional methods for quantifying body chemistry generally involve sampling blood, tissue, or urine. Advances in analytical instrumentation now enable an additional modality, breath sampling, to be performed. It is now possible to non-invasively sample and collect on an adsorbent virtually all of the volatile materials that an individual exhales. This adsorbent can then be submitted for analysis to provide a unique perspective on the composition of exhaled breath. The results from these analyses are hypothesized to provide the study subject's general status.

To achieve this goal, an apparatus to collect breath that is accurate and convenient has been developed. The basis of capture is an adsorbent finely tuned to trap the analytes of interest. Theoretically, more than one type of chemistry could be employed. Selection of an adsorbent is based on the availability of adsorbents that are able to collect all the organic analytes or those that preferentially select specific analytes. Depending upon the particular application, a plurality of adsorbent tubes may be used. For example, a universal tube requires longer collection times than one fine-tuned primarily for low molecular weight alkanes.

We have focused on the development and validation of protocols for the collection of representative breath samples from spontaneously breathing human subjects and laboratory rodents. We have designed independent sampling protocols for the collection of exhaled breath from human subjects and laboratory rodents. The protocol for human subjects allows minute ventilation and carbon dioxide to be measured continuously during breath collection, whereas the protocol for laboratory rodents involves quantifying only continuous carbon dioxide during collection. These novel protocols are crucial for the development of sensitive, real-time, non-invasive biomarkers in exhaled breath. Additionally we have evaluated glass tubes packed with well-defined adsorbents as a new way to collect breath. These new breath collection protocols have been validated using human subjects and laboratory rodents and the results compared to those obtained from breath collected in inert gas sampling bags. In studies to date, we have been able to quantify subtle effects of lifestyles, diet and exercise on exhaled breath. Our new approaches provide significantly greater number of potential biomarkers (including reactive molecules) for future studies.

Breath Collection and Analysis

Samples of breath are collected after the human subject appears to be breathing normally. A biological filter in-line on the mouth port of the one-way non-rebreathing valve and a disposable mouthpiece is used during breath collection to prevent contamination of the breath-monitoring equipment with infectious agents. These procedures exceed recommended pulmonary function test safety practice. A portion of the exhaled breath is sampled isokinetically at 90 ml/min for 1 minute from the

expiratory port of the one-way non-rebreathing valve using the battery-operated commercial ambient air sampler.

Conversely, laboratory rodents are placed in specially designed glass chambers and ultrapure air flowed through the chamber for a defined time to equilibrate the chamber and rodent to the ultrapure air. After this period of equilibration, a portion of the out flowing gas is sampled isokinetically at 90 ml/min for 1 minute using the battery-operated commercial ambient air sampler.

Breath (or outflow gas) is collected on duplicate commercial multibed adsorbents packed in glass thermal desorption tubes. Each thermal desorption tube has a unique number engraved on the tube. This number is used to track each sample of exhaled breath. The thermal desorption tubes are packed with unique adsorbents Carbopack X and Carboxen 1018. Carbopack X, a graphitized carbon with a surface area of 240 m^2/g, is a weak adsorbent that adsorbs all compounds greater than about C4 or C5. Carboxen 1018, a carbon molecular sieve with a surface area of 700 m^2/g, is a strong adsorbent with defined porosity that adsorbs compounds C1 through C5. These proprietary adsorbents were developed in collaboration with Supelco Inc. of Bellefonte, Pennsylvania. The breath samples are drawn through the weak adsorbent bed followed by the strong adsorbent bed. This design of adsorbent bed allows all the molecules of interest to be collected and subsequently thermally desorbed for analysis by capillary gas chromatography. After the sample has been collected, the thermal desorption tube are capped and stored until analyzed. Research has shown that breath and air samples are stable in thermal desorption tubes for at least a year, although all samples are analyzed as soon as possible after collection.

Results

The collected breath samples are analyzed using two-stage thermal desorption capillary gas chromatography. See figure 26. Preliminary identification of breath analytes is made on the basis of comparison of the retention volume for the unknown molecule with those of known standards. Confirmation of these identities is made using secondary methods of identification such as electron impact mass spectrometry. Quantification of breath analytes is made from calibration curves for known concentrations of molecules.

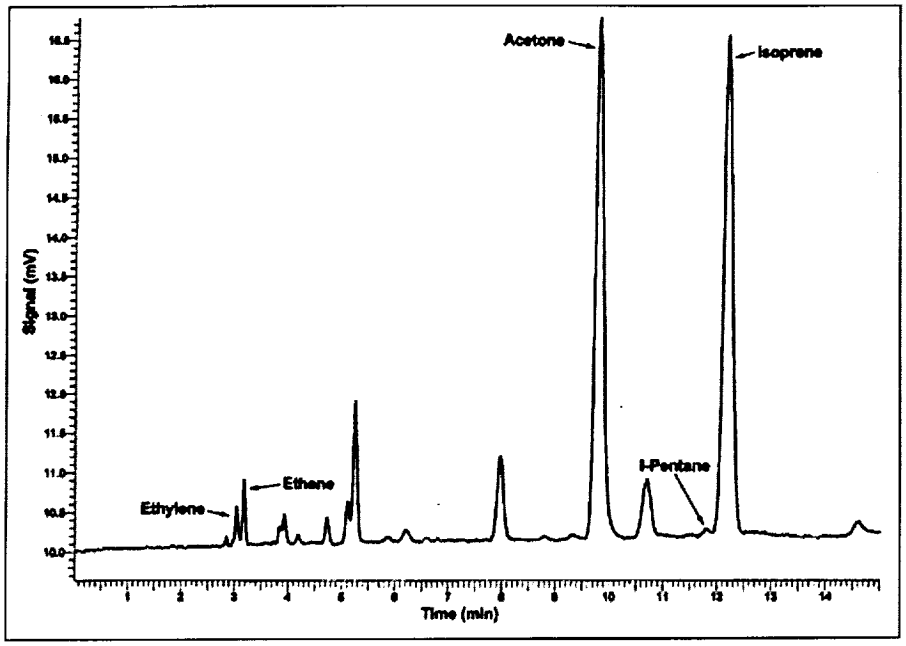


Figure 26. Capillary gas chromatograph of a representative breath sample collected and analyzed using thermal desportion tube.

In previous studies we have demonstrated several different types of acute pro-oxidant tissue injury that provided significant evidence that breath ethane is a sensitive, reproducible biomarker of oxidative stress status in humans. We have also mechanistically accounted for the pathways that lead to ethane production in these pro-oxidant situations. We have correlated increases in the levels of breath ethane to levels of blood and we have tissue biomarkers of oxidative injury. Moreover, we have biochemical quenched the pro-oxidant response and shown that this effect was paralleled by the changes in breath ethane.

In case-controlled studies, designed to better dissect the relationship between dietary antioxidant status and breath ethane levels in adults, we have showed that there was a direct correlation between cigarette smoking (external stress factor) and several biomarkers of increased oxidative stress, including increased levels of breath ethane.

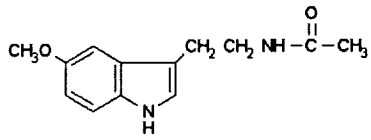
We have explored breath ethane as a biomarker of the dietary-induced pro-oxidant processes that induce atherogenesis in normal adults. During a controlled human feeding study that was designed to limit dietary fat intake and increase antioxidant consumption (via increased fruit and vegetable consumption) we found that dietary modification in ways that increase healthy lifestyle factors significantly affect serum antioxidant capacity. Importantly, this response was protective against oxygen-free radical lipid peroxidation.

We have validated our breath sampling protocol that continuously measures and records the instantaneous gas flow, concentration of carbon dioxide and mouth pressure as a function of time during breath collection with study subjects in a controlled laboratory setting. This setting has allowed the collection of comparable information for the rate of perfusion in the pulmonary vasculature during breath

collection. The following cardiac parameters were collected continuously during breath collection: heart rate, blood pressure, ejection fraction, peak emptying rate, peak filling rate, end diastolic volume, end systolic volume, systolic volume, and cardiac output. Additionally, carbon dioxide production, oxygen consumption, and anaerobic threshold were quantified continuously.

The combination of all this information has enabled the composition of exhaled breath to be related to normal physiology, which is a prerequisite for an understanding of the excretion of endogenously produced analyte molecules. This study provided unique information on how these parameters varied during well-defined exercise. The relationship between exercise and non-invasive testing protocols has major relevance to the mission of the space program.

Melatonin in Breath using Matrix-Assisted Laser Desorption Mass Spectrometry
One of the major objectives of the NSBRI Human Performance Factors, Sleep and Chronobiology Team is to develop strategies to monitor the circadian physiology of astronauts during long-duration space missions. The Team has identified that there is a critical need for in-flight assessment of melatonin levels. Melatonin is recognized as a very reliable marker of the human circadian pacemaker. It is an amino acid derivative secreted by the pineal gland during the night. It plays an important role in the regulation of the circadian sleep-wake cycle. It is a more reliable indicator than core-temperature measurements. As an indicator of the state of the human circadian pacemaker, it would be desirable to monitor, at intermittent times, the astronauts' plasma or salivary levels of melatonin.



Chemical Structure VII of Melatonin (N-acetyl-5-methoxytryptamine.)

Recent studies have indicated that there is very reliable correlation between the salivary and plasma levels. Because the sampling of plasma melatonin is an invasive procedure, it would be desirable to have a means of measuring salivary melatonin in subjects on long-duration space missions.

We have performed a preliminary analysis of salivary melatonin using MALDI time-of-flight mass spectrometry of melatonin in saliva. Mass spectrometry may provide a reliable, convenient, and economical way to track melatonin during space missions. Gas chromatography - mass spectrometry is regarded as the standard for quantitative identification of melatonin in biological fluids because it is highly sensitive and selective. It is not used routinely for sample analysis because of the high cost of the instrumentation.[16]

Melatonin was detected by its molecular weight using the matrix-assisted laser desorption/ionization mass spectrometer. A human saliva sample (sample obtained at 2:00 AM) was filtered through a carbon cartridge (Waters Oasis HLB cartridge) with the use of a vacuum. This filtering was done in the normal fashion, condition with methanol, equilibrate with water, load with sample, wash with 5 % methanol in water, and elute with methanol. The wash is dried down and re-dissolved to yield a concentration that is detectable with MALDI-MS. The sample was then mixed 50/50 with matrix, DHB, and laid down on the mass spectrometer slide. A comparison of matrix (DHB), melatonin and human saliva is shown in figure 9. The molecular weight of melatonin is 232.3 Da. Dilution studies and HPLC analysis of melatonin in saliva will be completed prior to publication of this work.

Other hormones that have been identified in saliva include cortisol, testosterone, estradiol, melatonin, progesterone, oxytocin, and androstenedione.

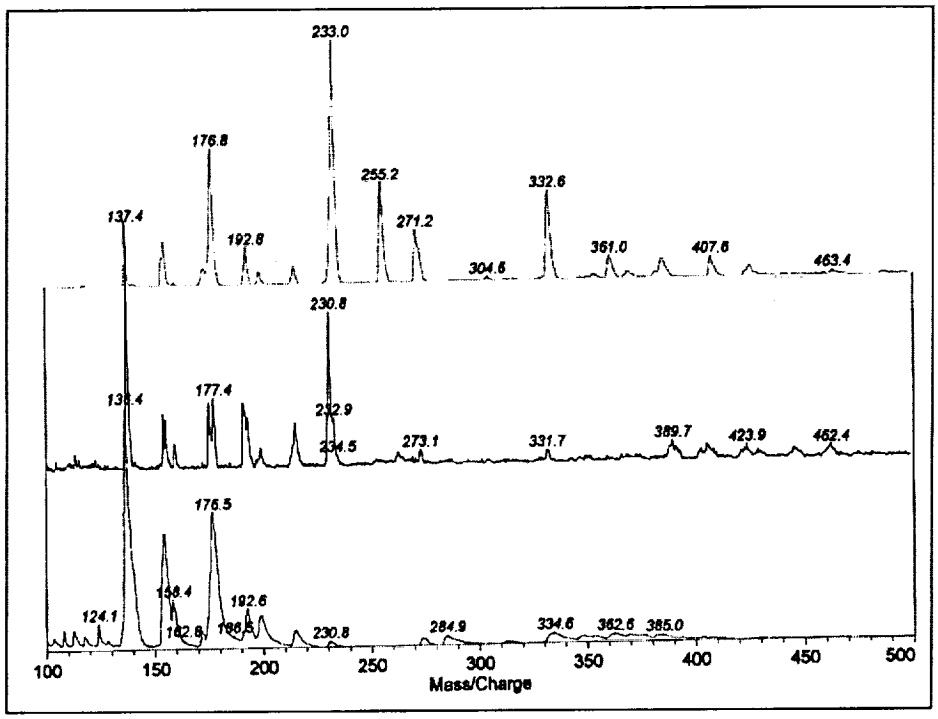


Figure 27. Mass spectra of human saliva in DHB (top), melatonin in DHB (middle), and DHB (bottom) compared.

F. Whole Blood

Whole blood is the biological fluid of choice for therapeutic drug monitoring and for performing pharmacokinetic studies. Several analytical methods have been developed for the detection and quantification of various medical biomarkers in blood using various extraction techniques involving time-of-flight mass spectrometry.[17] [18][19] Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) has proven to be sensitive as well as selective techniques in therapeutic drug monitoring. The limit of detection (LOD) and limit of quantification (LOQ) for mass spectrometry alone are in the single digit ng/mL range, respectively. Blood samples are routinely analyzed in conjunction with other analytical techniques such as fluorescence polarization immunoassay (FPIA), or high-performance liquid chromatography (HPLC).

Certified human serum was obtained from New York Biologics, Inc., USA. The serum was from a pooled source. It was kept frozen until analyzed. The samples were allowed to equilibrate at room temperature for about 4 h before testing. The spectrum of pooled whole blood was obtained with no preprocessing (chromatography). The blood was diluted with water to a 1:1 ratio.

The spectrum in Figure 28 was recorded in DHB matrix. The spectrum of whole blood plasma in cyano-4-hydroxycinnamic acid matrix is shown in figure 29. These spectra exhibit well-defined peaks between 100 to 400 mass units. The spectrum above 400 m/e is not easily observed.

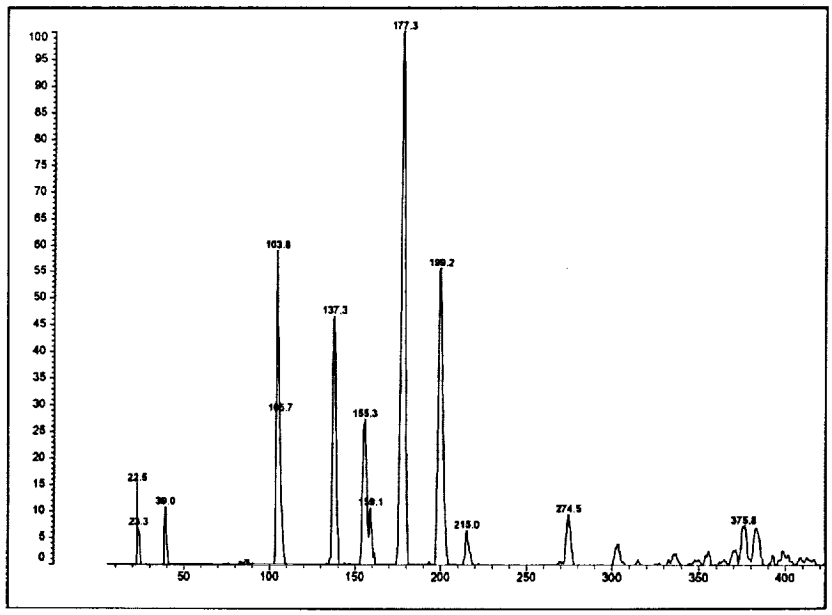


Figure 28. Spectra of pooled blood plasma in DHB matrix.

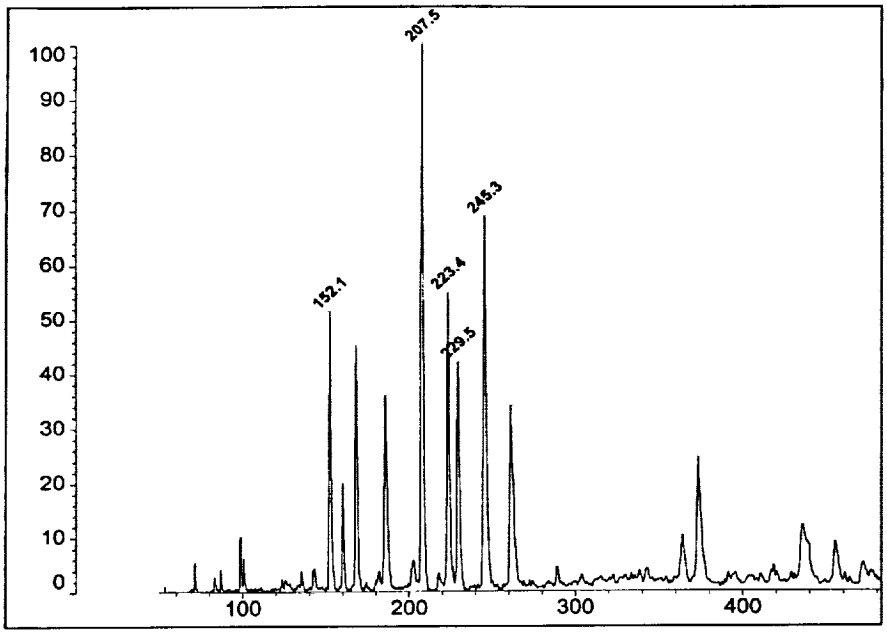


Figure 29. Spectrum of whole blood plasma in cyano-4-hydroxycinnamic acid matrix.

Three different approaches were chosen to prepare the blood serum to elucidate high molecular weight components. Treatment of whole blood with NH_4HCO_3 and dilution gave higher molecular weight compounds. We showed that modification of blood by additives render it detectable by MALDI. The salt additives solubilize the hydrophobic components. Studies of the sample preparation will be required to show the various components of blood.

Whole human blood was diluted by a factor of 1000 with 100mM ammonium bicarbonate (pH = 8). The matrix was prepared by dissolving 10mg/ml α-cyano-4-hydrocinnaminc acid in a 2:1 acetonitrile : 0.1% trifluoroacetic acid solution. The blood-ammonium solution was then further diluted by adding one part of blood-NH_4HCO_3 to nine parts of matrix. Two micro liters of sample solution was placed onto the MALDI cell and allowed to evaporate prior to analysis. Molecular weights over of 5,000 m/e have been reported by this method.[20]

G. Radiation Effects: Early Detection of Cancer Peptide Biomarkers

The risks to personnel in space from the naturally occurring radiation are generally considered to be one of the most serious limitations to human space missions. The NSBRI is examining the consequences of radiation in space *in vivo* in order to develop countermeasures, both physical and pharmaceutical, to reduce the risks of cancer and other diseases associated with such exposures. The consequences of exposure to radiation in space are considered a major limiting factor for long-duration interplanetary space travel for humans. Radiation doses in space may be hundreds of times greater than those experienced on earth. These energetic charged particles (protons, helium and iron ions), as well as secondary types can kill cells in the body or cause mutations that may lead to cancer, cataracts, central nervous system damage or other diseases.

We evaluated three novel peptide cancer biomarkers to demonstrate the utility of MALDI-TOF as a tool for the early detection of carcinomas. The biomarkers were obtained from the University of Arkansas under a confidentiality agreement. The University of Arkansas is looking for biomarkers for the early detection of ovarian cancer.[21] Ovarian cancer is the number one killer of women with gynecological disease. Despite the development of diagnostic assays based on the ovarian cancer antigen CA125, the disease is not diagnosed until its late stages, greatly reducing the chance of survival. Recent research has lead to the identification of other possible candidate markers, such as serine proteases, which are present in the earlier stages of the disease. Three new diagnostic peptide fragments were analyzed using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectroscopy.

For malignant cells to grow, spread, or metastasize, they must be able to invade local host tissue and dissociate from the primary tissue. They must enter and survive in the bloodstream, implant into the surface of a target organ, and establish an environment conducive for growth, which includes the induction of angiogenic and growth factors. Extracellular proteases are believed to promote all of these actions, thereby implicating them in the growth, spread and metastatic progression of many cancers. This also implies that extracellular proteases may be candidates as markers of carcinoma development. The serine protease known as PSA (prostate-specific antigen) has been used as a tumor marker for the early diagnosis of prostate cancer, due to its abnormal prevalence in the peripheral blood of patients.

Using differential display PCR (polymerase chain reaction) amplification comparing tumors and normal ovary, three gene products (serine proteases) have been found which fulfill requirements to become potential candidates for ovarian carcinoma detection. These requirements include significant overexpression in carcinomas relative to normal ovarian tissue in early stage disease (stage I-II); high expression in all tumor subtypes; either secretion signal sequences or transmembrane sequences confirming potential for export into extracellular space and uptake into the circulatory system; confirmed presence in malignancy but lack of presence in normal adult tissue; and confirmed protease protein in both cultured tumor cell lysates and tissue culture medium. These criteria suggest a direct relationship between tumor presence and protease presence in serum and/or urine of patients with ovarian cancer.

Three gene products have been identified.[22] These proteases, once activated in the body, will be metabolically destroyed by proteases of the circulatory system, giving rise to peptide fragments. Such peptides would be present in the blood and may be passed through the glomeruli to the urine of patients. Proteolytic activation of each of these enzymes results in the release of propeptides (Peptide-A, Peptide-B, and Peptide-C). MALDI-TOF analysis of synthesized, purified forms of these activation peptides is the initial step to define a protocol for identification of these tumor markers in blood/urine.

Sample Preparation. Sample preparation is critical for MALDI analysis of biological samples.[23] Two steps are generally necessary for analysis: 1) isolation and purification of sample, and 2) choice of matrix and analyte concentration, pH adjustment, crystallization conditions, and use of additives and on-target sample clean-up. Because the samples were provided in a near-neutral solution, pH adjustment was not necessary, and additives were not utilized. Purification, matrix selection, and on-target sample cleanup were investigated.

Purification of a sample can be a challenging process. While many groups have demonstrated that MALDI-TOF can be used to analyze synthetic (pure) peptides, analysis of peptides extracted from biological sources is not as routine. Even moderate concentrations of salts, glycerol, and detergents used for sample extraction detract from the desorption/ionization efficiency of peptides, reducing mass range and resolution. One common purification technique, HPLC (high performance liquid chromatography), often results in thirty- percent sample losses and can contaminate samples. Sample purifications that offer more merit include membranes, on which impurities may be washed away; drip columns; and Millipore ZipTips. Both drip columns and ZipTips make use of membrane surfaces. A primary focus of this research was to determine a useful sample preparation technique to purify these peptides that were supplied in a phosphate buffer saline.

Procedures

Synthesized activation peptides were provided by the University of Arkansas for Medical Sciences (from Bio-Synthesis, Inc.). Samples were received as 1mg/ml solutions in 95% PBS and 5% DMSO, and as dehydrated solids, purified via C18 column (peptide presence verified via absorbance reading at 214nm). The determined molecular weights were the following: TADG-14 Activation peptide (NH2-GHSRAQEDK-OH) MW = 1026.07; Protease M Activation peptide (NH2-AEEQNK-OH) MW = 716.74; Peptide- Activation peptide (NH2-EEAQGDK-OH) MW = 774.28. (All MALDI-TOF analyses were performed using a Kratos Kompact MALDI IV mass spectrometer, in linear, positive mode. Laser power ranged from 55-75, and 50 profiles were performed per analysis.

Matrix Selection. Five matrix solutions were prepared: saturated solution of ferulic acid (FA; trans-4-hydroxy-3-methoxycinnamic acid; MW 194.19) in 30:70 CAN (acetonitrile): H_2O/0.1%TFA; saturated solution of sinapinic acid (SA; 3,5-dimethoxy-4-hydroxycinnamic acid; MW 224.21) in 70:30 ACN:H_2O/5%TFA; 50mM SA solution in 70:30 ACN:H_2O/0.1%TFA; 150mM alpha-cyano-4-hydroxycinnamic acid (CHCA; MW 189.17) in 70:30 ACN:H_2O/0.1%TFA; and a saturated solution of 2,5-dihydroxybenzoic acid (DHB; MW 154.12) in 70:30 ACN:H_2O/0.1%TFA.

Calibration. External mass calibrations were performed using Angiotensin II (MW = 1046.2) in varying matrices. Calibrant was deposited in probe wells (0.5µL samples), followed by an equal addition of matrix while still wet. Matrix water loss and ionized angiotensin ($M+H^+$) peaks were used for calibration.

Purified Peptides. Each dehydrated peptide was dissolved in 5µL of 0.1%TFA in water. 0.5µL peptide samples were deposited into wells, followed by 0.5µL of 150mM CHCA in 70:30 ACN: H2O/0.1%TFA while the peptides were still wet. Pulsed extraction was used for each peptide run (PE = 1024 for Peptide-B, 716 for Peptide-C, and 773 for Peptide-A). Matrix blanks were also run. Reflectron mode was also utilized for these peptides.

Peptides in PBS/DMSO: Membranes. A PVDF (polyvinylidene fluoride) membrane was taped to a probe and pre-wet with methanol. While the membrane was still wet, 0.5µL of peptide solution was deposited. After drying, 0.5µL saturated CHCA matrix solution (70:30 ACN:H_2O/5%TFA) was deposited. The samples were analyzed using pulsed extraction as before. Matrix blanks and external calibrations with Angiotensin II were also analyzed on the membranes (calibrating on angiotensin II and through zero).

Peptides in PBS/DMSO: Oasis HLB sorbent. An *Oasis HLB* sorbent (Waters Corp.) was also used to analyze the peptides. The *Oasis* membrane was pre-treated with 1ml methanol, followed by 1 ml deionized water. 0.25ml of the Peptide-B solution was loaded and washed with 0.25ml of 5% methanol in water. 1ml methanol was used to elute. The sample was placed in a Speedvac to evaporate the methanol. 5µL of 0.1%TFA in water was then added to reconstitute the purified sample. 0.5µL of peptide was placed onto probe wells and allowed to dry. 0.5µL saturated CHCA in 70:30 ACN: H_2O/5%TFA was then deposited. Pulsed extraction was used (set to 1024).

Peptides in PBS/DMSO: Millipore ZipTip$_{C18}$. ZipTip procedures were utilized for the Peptide-B and the Peptide-C samples in PBS. 10µL of 0.1%TFA in water was mixed with 5µL of sample to maximize analyte binding potential. The ZipTips was pre-wet with a 10µL wash, repeated, of 70:30 ACN:H_2O/0.1%TFA. The tip was then washed twice with 0.1%TFA in water for equilibration. Ten aspiration/dispension cycles of the sample/TFA solution through ZipTip were performed. 2µL of elution solution (70:30 ACN:H_2O/0.1%TFA) was dispensed into a clean vial. Aspiration/dispension of the eluant through ZipTip was performed three times without introducing air. The purified sample was directly dispensed onto the target probe and allowed to dry. 0.5µL saturated CHCA (in 70:30 ACN:H_2O/5%TFA) was then deposited onto the sample and dried. Matrix blanks were run, and calibrations with Angiotensin II were performed, all utilizing pulsed extraction.

Results

Selection of an appropriate matrix and calibrant was performed first. Angiotensin II was selected as the calibrant because of its molecular weight (1047), which is larger than but close to the largest peptide sample (Peptide-B, 1026). Out of the matrices run for selection, DHB and CHCA produced good results with the Angiotensin II (see Figure 30). CHCA was chosen because it yielded the best signal in the low-mass region of the samples.

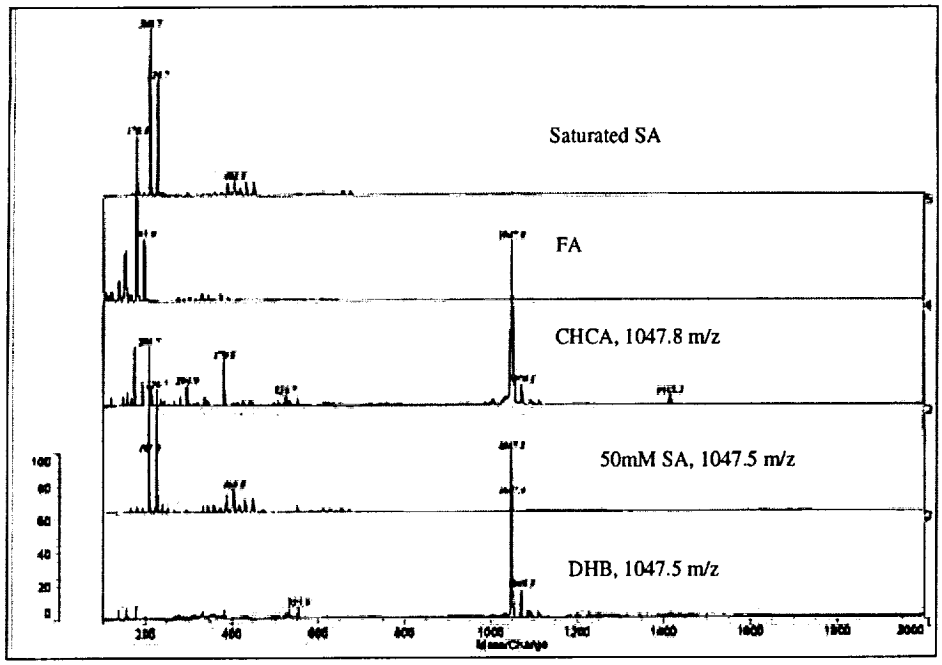


Figure 30. Matrices and Angiotensin II

The dehydrated peptides, purified via C18 column, were loaded and ionized using pulsed extraction, set to the expected peak value for each sample. The largest peptide solution, Peptide-B, showed the greatest signal strength of the three, at a peak of 1026m/z. The Peptide C solution showed a peak at 717m/z, and Peptide-A solution showed a peak at 773m/z. The peaks were not strong (3.8mV-26mV), and they were very noisy. See Figures 31-33. The reflectron mode was utilized in an attempt to augment resolution, but the signal was not improved. These results were not clear enough to say with definitely that the samples were present. These results also suggest that the HPLC C18 column purification process may not be ideal.

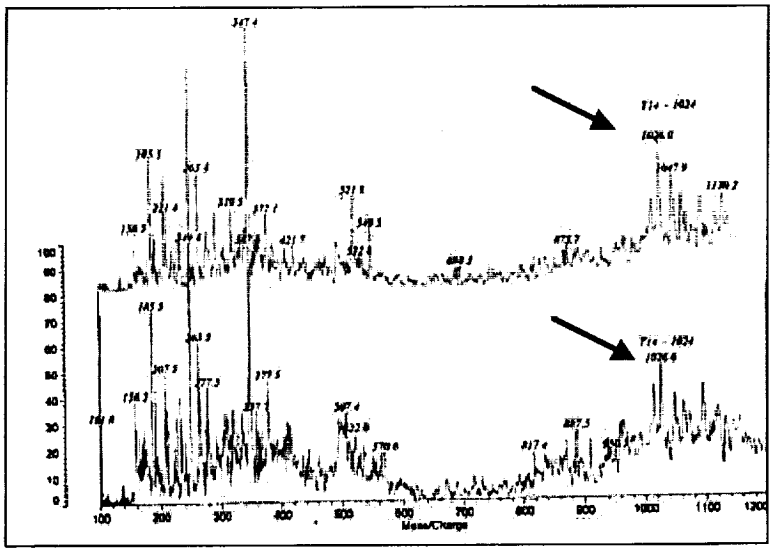


Figure 31. Peptide-B sample purified by C18 column. Arrows indicate peaks at 1026 m/z.

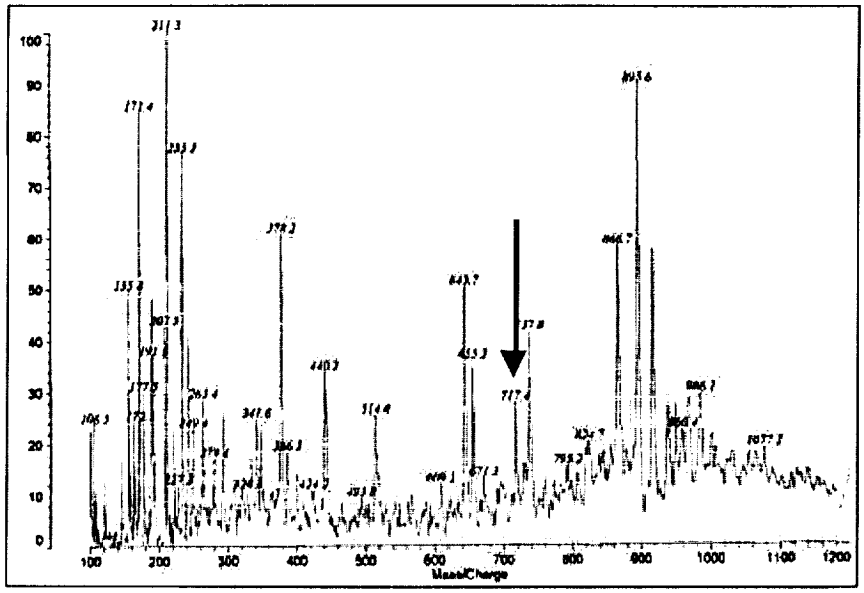


Figure 32. Peptide-C sample purified by C18 column. Arrow indicates peak at 717 m/z.

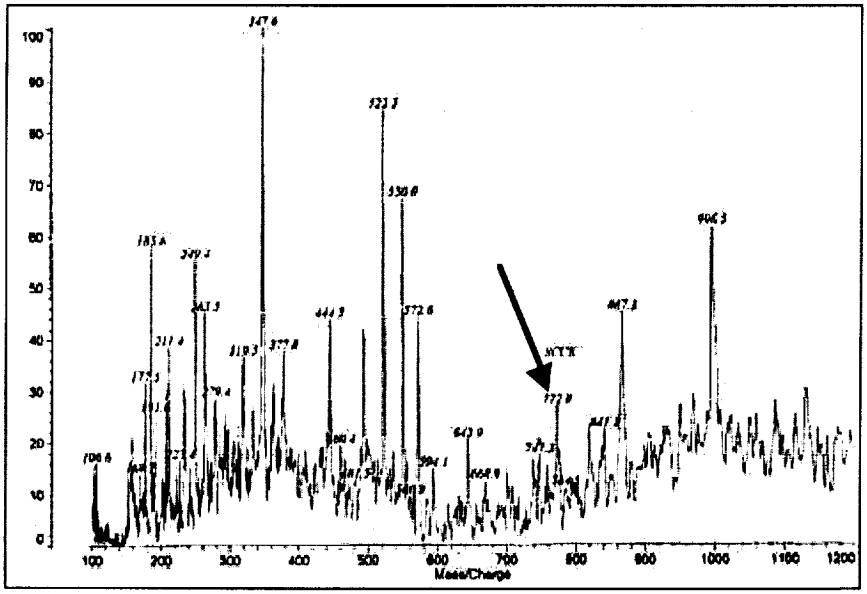


Figure 33. Peptide-A sample purified by C18 column. Arrow indicates peak at 773 m/z.

The non-purified Peptide-B and Peptide-A solutions were also purified using Millipore ZipTips$_{C18}$. The Peptide-B sample showed a peak at 1028 m/z (see Figure 34), while the SCCE peptide still showed no significant peaks.

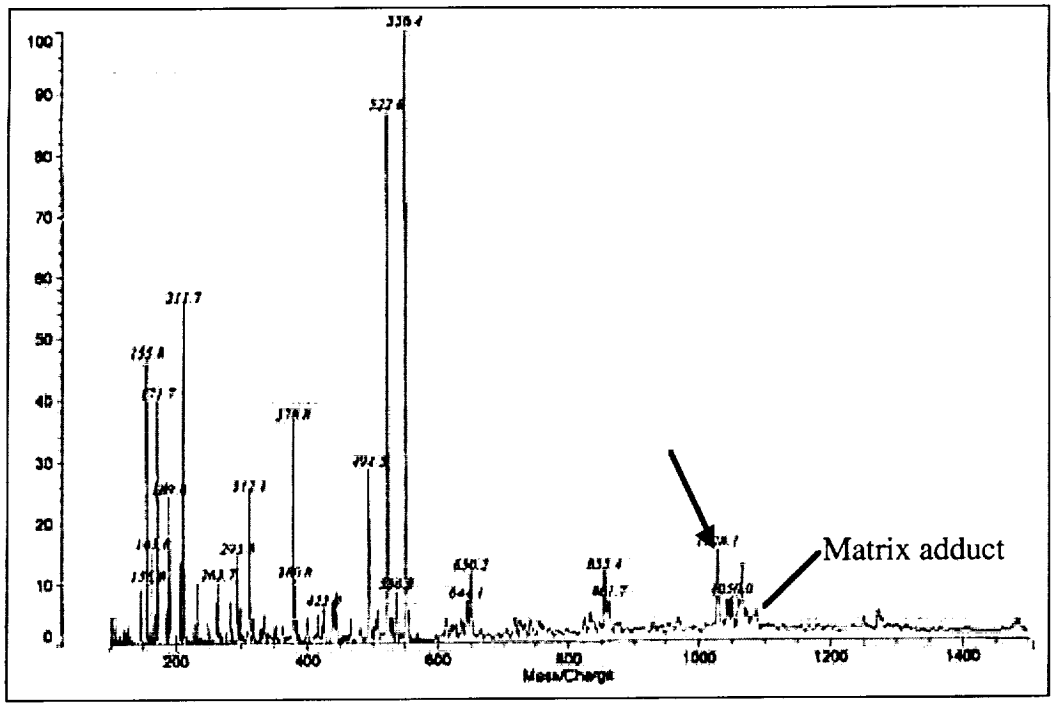


Figure 34. Peptide-B sample purification with Millipore ZipTip. Arrow indicates peak at 1028 m/z.

Sample preparation techniques are critical for analyzing complex biological fluids such as blood and urine. The peptides examined in this study were delivered in a high-salt solution, requiring preparation techniques similar to those required for biological fluids. A number of techniques were tested, including HPLC C18 columns, PVDF membrane, a drip column, and the Millipore ZipTip$_{C18}$. The HPLC purified peptide results were extremely noisy, signifying low sample presence, if at all. The Peptide-Activation peptide and the Protease M activation peptide were detected using a PVDF membrane, although characteristic peaks for the peptide could not be observed. The drip column results did not indicate sample presence. Finally, the Zip-Tip purification seemed to work for the Peptide-Activation peptide, but not for the SCCE peptide. Based on these results, it can be concluded that MALDI-TOF mass spectroscopy can be used successfully for the detection of the ovarian cancer biomarker peptide fragments. However, purification techniques are necessary. Further work utilizing PVDF membranes and Millipore ZipTips should be pursued to develop a protocol for definitive MALDI-TOF detection.

Competing diagnostic tools for early detection of carcinoma are currently in development. The advantage in using MALDI-TOF for detection over these other methods is the robust nature of the analyzer. MALDI-TOF is rapid, sensitive, and tolerant of salts in biological samples, MALDI-TOF tumor biomarker detection is not limited to ovarian carcinoma. The identification of other tumor biomarkers identified by NSBRI research teams may be performed using MALDI-TOF.

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II. IMPLICATIONS OF PROJECT FINDING FOR FURTHER RESEARCH

Successful development and testing of the "Miniature Time-of-Flight (TOF) Mass Spectrometer" will provide NSBRI/NASA with a complete medical diagnostic system to measure biochemical **biomarkers** for bone, internal organs, and soft tissues, routinely and non-invasively. It will also serve to evaluate the effectiveness of applied **countermeasures** to the effects of space travel. This compact medical diagnostic system will provide an autonomous and semi-autonomous patient monitoring system with a low false positive alarm rate.

Using a grant from the NSBRI, The Johns Hopkins University Applied Physics Laboratory has completed initial laboratory studies with critical biomarkers for muscle, cardiac and bone atrophy. The NSBRI project has recorded full spectrum mass spectral signature of many key target biomarker analytes using the MALDI technique at physiological concentrations.

The objective of this technology feasibility project was to lay the scientific and engineering foundations to design, build and launch a flight qualified "Miniature Time-of-Flight Mass spectrometer" (TOFMS) for use on space platforms such as the Space Shuttle and the International Space Station (ISS). Successful deployment of this instrument in near-earth missions will establish the medial, engineering and scientific groundwork to adopt this medical diagnostic instrument for a mission to Mars later this century.

The current laboratory working model of The Johns Hopkins TOFMS meets many of the stringent requirements necessary to be utilized in space. The instrument is small (less than one cubic ft), lightweight (less than 5 kg), low power (less than 50 watts), and it is a rugged device that can be used continuously with advanced signal processing diagnostics. To date, we have recorded spectra of compounds from under 100 to beyond 10,000 atomic mass units (amu). This atomic mass range covers the molecular weights of most common biological biomarkers. Further, the output of the TOFMS makes it ideal for autonomous and semi-autonomous patient monitoring, as well as rapid telemetry to earth for in-depth analysis by ground support teams. In its present form, it can be readily modified for human space applications.

The Countermeasure Readiness Level (CRL) developed by NASA describes the level of scientific maturity of applied research from the development of a hypothesis to validated procedure ready for operational implementation of procedures and devices that can mitigate the deleterious effects on humans engaged in space flight. Using this scale as a metric, this project was at Level 5, "Proof of concept testing and initial demonstration of feasibility and efficacy." Based on the work completed to date, we believe that this project successfully met the goals of a level 5 CRL.

Based on the current state of the instrument design and the analysis of biomarkers that we have completed to date, the project can successfully transition from feasibility study to a countermeasurement development program.

A follow-on countermeasurement development program should include the following tasks:

1. A prioritization and down selection of specific biomarkers and biochemical countermeasures (essential to evaluate crew health) must be made. Based on this prioritization and selection process, efforts can be focused on a limited set of biomarkers.

2. Sampling and sample preparation techniques protocols need to be established to enable the MALDI TOF mass spectrometer system to reliably detect, identify and quantify extremely low levels of chemical and biological substances in complex body fluids (urine, blood, and breath).

3. The results of this effort should be closely coupled to ongoing NSBRI, NASA, and industry research and development efforts where specific biomarkers are being evaluated in a clinical laboratory facility using established assays.

4. Design, develop and test a fast, portable gas chromatograph – time-of-flight mass spectrometry (GC-MS) system for human spaceflight applications. It will provide complementary information to the MALDI method. It will also reduce the amount of preprocessing that will be required to measure biomarkers in breath, blood and urine.

APPENDICES

A. Project Research Data

Project research data is presented in the body of the final report. The large volume of additional mass spectral data recorded over the past three years is stored electronically on a hard drive with the time of flight mass spectrometer at APL Building 2 room 117. An electronic copy of this data will be sent to NSBRI on disk.

B. Oral Presentation and Conference Paper

"Real Time Sampling and Analysis of Biological-Chemical Aerosols by TOF Mass Spectroscopy" will be presented in the technical session Medical Aspects of Environmental Monitoring at the 28th International Conference on Environmental Systems, July 13-16, 1998 at Danvers, Massachusetts.

Oral Presentation, "Measurements Of Human Performance Biomarkers Using Time-Of-Flight Mass Spectrometer," Space Technology and Applications, International Forum, Albuquerque, NM, January 1999.

M. D. Antoine, "Mass Spectrometry in Human Spaceflight," Colloquium of The Johns Hopkins University Applied Physics Laboratory, 1999. (Invited).

Meeting Abstract and Oral Presentation, "Quantitative Analysis of 3-Methylhistidine in Biological Fluids by Matrix-Assisted Laser Desorption Mass Spectrometry," Miquel D. Antoine, Richard S. Potember, and Wayne A. Bryden, 5th Research and Development Symposium, The Johns Hopkins Applied Physics Laboratory, November 2, 1999.

Oral Presentation: "Miniature Time-Of-Flight Mass Spectrometer," The Space Technology and Applications International Forum (STAIF-2000) January 30-February 3, 2000.

Invited Presentation: "Study of Melatonin Using the Miniature Time-Of-Flight Mass Spectrometer," Circadian, Neuroendocrine And Sleep Disorders Section, Harvard Medical School, April 7, 2000.

Meeting Abstract and Oral Presentation: "Real Time Analysis Of Bone Demineralization / Calcium Metabolism Biomarkers Using A Miniature ToF Mass Spectrometer," The 13th IAA Humans in Space Symposium, Greek Aerospace Medical Association, May 20-26, 2000, Santorini, Greece.

JHU/APL Invention Disclosure: TOF Mass Spectrometry to Monitor Human Bone Resorption Using Type-I Collagen Cross-linked N-telopeptides in Urine, September 2000.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Final Report

September 2000

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Executive Summary

High-energy charged particles of extra-galactic, galactic and solar origin collide with spacecraft structures in Earth orbit outside the atmosphere and in interplanetary travel beyond the Earth's magnetosphere. These primaries create a number of secondary particles inside the structures that can produce a significant ionizing radiation environment. This radiation is a threat to long term inhabitants or travelers for space missions and produces an increased risk of cancer and DNA damage. The primary high energy cosmic rays and trapped protons collide with common spacecraft materials such as aluminum and silicon and create secondary particles inside structures that are mostly protons and neutrons. Indeed, the effect of tens of grams per square centimeter of structure or atmosphere is to convert and multiply the primary proton "beam" into a secondary environment dominated by neutrons between several MeV and several tens of MeV. Charged protons are readily detected and instruments are already in existence for this task. Neutrons are electrically neutral and therefore much more difficult to measure and detect. These neutrons are reported to contribute 30-60% of the dose inside space structures and cannot be ignored. Currently there is no compact, portable and real time neutron detector instrumentation available for use inside spacecraft or on planetary surfaces where astronauts will live and work.

Present neutron detection systems use gas tube proportional counters for the monitoring of low energy (0.025 eV to 1 MeV) neutrons. However for higher energies the detector systems are quite large and massive and often employ passive detection methods that must be recycled and read out after the fact. Physically large neutron diffraction tables are used for accelerator experiments. Emulsions are flown on the Space Shuttle and returned to Earth for analysis. The NASA Ames aircraft uses an instrument built with Bonner spheres which are large spheres of polyethylene moderator some tens of centimeters in diameter with a photodiode in the center and weighing 1500 pounds.

In 1997 we proposed to design and build a portable, low power and robust neutron spectrometer that measures the neutron spectrum from 10 KeV to 500 MeV with at least 10% energy resolution in the various energy intervals. This instrument will monitor the existing neutron environment both inside spacecraft structures and on planetary surfaces to determine the safest living areas, warn of high fluxes associated with solar storms and assist the NSBRI Radiation Effects Team in making an accurate assessment of increased cancer risk, DNA and central nervous system (CNS) damage to astronauts. The instrument uses a highly efficient proportional counter Helium 3 tube at the lowest energy intervals where equivalent damage factors for tissue are the highest (10 KeV-2 MeV). The Helium 3 tube is shielded with a cadmium absorber to eliminate the much less damaging and, hence, uninteresting, but more prevalent, thermal and epithermal neutrons and to make the structure of the spectrum more accurate in the 20 KeV-2 MeV range. A second option is to use a pair of tubes, one shielded and one unshielded, combining the difference in their counts to yield the thermal neutron contribution. The spectrometer also uses a 5mm lithium drifted bulk silicon solid state detector in the neutron energy range of 2-500 MeV due to its demonstrated and modeled detection efficiency of 3-5% in the 5-150 MeV energy range. In high energy regions equivalent damage factors for dose

equivalent are lower but hits from one or a small number of neutrons may prove to be important in sensitive localized volumes. The silicon detector system for high energy neutrons will discriminate against charged particles by using a plastic cesium iodide scintillator of an appropriate geometry (a small cup and plug configuration for a Mars Lander; a surrounding rectangular liner for a Space Station Express Rack) monitored by a silicon PIN photodiodes.

The first round of experiments with monoenergetic neutron beams on the Helium 3 tube and 5mm silicon detector systems were performed in February and June 1999. Both detector systems have previously been evaluated with Californium (mean energy ~ 1 MeV) and Americium/Beryllium (mean energy ~5 MeV) radioactive neutron sources at NIST. The Helium 3 tube exhibited energy resolution of at least 1 KeV over the energy range from 10 KeV to 3 MeV. The efficiency of detection of the tube decreased from 80% at energies of tens to hundreds of KeV to 0.1% as 1 MeV was approached as would be expected from the behavior of the neutron capture cross section for the neutron-Helium 3 reaction over this energy range. The best performing high-energy silicon detector from the set of FY 1999 tests proved to be the 5mm thick lithium drifted silicon detector. It demonstrated surprisingly good efficiencies of 1-5% at the Columbia RARAF (Radiological Research Accelerator Facility) for neutron beam energies of 2.46, 5.89 and 14, 16.25 and 18.5 MeV.

The 5 millimeter thickness of the silicon detector is a reasonable fraction of the neutrons' mean free paths in silicon in the 2-150 MeV energy range thereby significantly increasing the probability of a neutron-silicon nucleus interaction. We plan to overlap the detection of neutrons with the Helium 3 tube in the 2-5 MeV energy interval for cross calibration purposes. In the energy region above 20 MeV the substantial progress that we have made with modeling and experiments in FY 1999 and 2000 has yielded a conceptual design of one or more thick bulk lithium drifted silicon detectors (5-7 mm in an array or ganged together to increase count rate or roughly double the detection efficiency respectively) surrounded by an appropriately configured charged particle anti-coincidence scintillator detection system. Results from simulations of any high-energy stack or proton recoil telescope configuration in January and March 1999 showed that only detection efficiencies of $5X10^{-4}$ would be achieved while maintaining 10% energy resolution for neutrons of energy greater than 50 MeV. Results from the RARAF experiments in February and June 1999 showed that the 5mm bulk silicon detector had as much as 5% ($5X10^{-2}$) efficiency between 10 and 20 MeV. Study of the Evaluated Nuclear Data Files (ENDF) archived by the Department of Energy showed that the total cross section for the neutron-silicon reaction remains fairly constant up to 150 MeV; thus, the bulk silicon detector efficiency is expected to do the same. The two order of magnitude advantage in detection efficiency for the bulk detector versus the recoil telescope disqualified the latter.

The late summer and fall of FY 1999 were occupied with two main activities: 1) our proposal to NASA in response to AO 99-HEDS-01 for a neutron spectrometer on the Mars 2003 Lander; and 2) the design and purchase of a set of detectors and associated electronics including battery packs for an engineering prototype instrument to be

qualified for aircraft flight. NASA/Dryden committed to several F 15/18 flights in January 1999 for our spectrometer to monitor avionics environment neutrons and help NASA check out our design.

The proposal for MANES (MArtian Neutron Energy Spectrometer) was submitted in August 1999. It was selected for a stage of further definition in November 1999 as a potential instrument for the then-scheduled Mars 2003 Lander. The status of this instrument and its funding has evolved from potential individual Mars 2003 Lander instrument (December 1999-February 2000), to possible combination with the JSC MARIE instrument after cancellation of the 2001 Lander in April (March-May 2000), to proposal for an extended definition phase for a 2005 Lander instrument after cancellation of the large 2003 Lander and selection of the two-Athena-Rovers-in-a-bag for 2003 in July 2000 (May-August 2000), to being on hold pending a decision to fly a large Lander in 2005. That next decision on the Mars Surveyor program is expected in late September/October 2000. The changing situation has required much communication, alertness, revised statements of work and costing in addition to the original proposal with both NASA Headquarters and Johnson Space Center (JSC). A grant of $123,000 was received from JSC for accommodation and definition phase work on MANES from February to August of 2000.

The efforts on the aircraft flight hardware were interrupted by the MANES proposal. Fabrication occurred in September-October 1999. Included in this two-month period were rise time discrimination experiments with the Helium3 tube detector at RARAF at the end of October. Pictures of the instrument fabricated for aircraft flight are included in Appendix A of this report and represent the engineering prototype hardware proposed and promised to NSBRI in 1997. The instrument actually exceeds the original expectations in that it has been packaged and qualified for an uncontrolled aeronautics environment. It is more than just a laboratory instrument.

The aircraft instrument was ready for qualification in November 1999 when our selection for the Mars 2003 Lander interrupted its progress again. Analysis of its mechanical integrity for flight vibration and acoustics did continue in the November 1999-January 2000 time frame. The results of the analysis required some strengthening of the instrument and an additional cover for acoustic dampening of the Helium3 tube. The actual vibration and temperature/altitude qualification test finally took place in February 2000. The instrument readily passed the vibration test and operated successfully at low temperature; however, an isolated corona breakdown occurred in one of the high voltage supply systems at a simulated altitude of 45,000 feet. The point of voltage break down was found and fixed and the instrument was successfully qualified to the simulated 45,000 feet. The instrument was delivered to NASA Dryden on March 23, 2000 and flown the week of April 24-28, 2000 on an F15 aircraft.

We took data up to 39,000 feet on ascent (the plane was to fly at a planned 40,000 feet cruise level) when we experienced a corona breakdown in the high voltage supply systems for both detectors. We are implementing and will implement more

comprehensive and robust fixes for each high voltage supply system and plan to repeat the aircraft flights when new funding is received in FY 2001.

In January 2001 we were notified that our proposal titled "Development of a Neutron Spectrometer to Assess Biological Radiation Damage Behind Spacecraft Materials" submitted in March 1999 in response to NASA NRA 98-Heds-05 would be funded for a period of 3.5 years from May 2000 to November 2003 at a level of $90,000 per year for a total of $315,000. Our primary responsibility under this grant is to support Lawrence Berkeley Laboratory (LBL) personnel in the evaluation of spacecraft structural and shielding materials by supplying a version of the neutron spectrometer compatible with ground-based accelerator research. Due to the unavailability of accelerator facilities our first scheduled test date is now January 2001.

The major effort in detector evaluation that took place in 2000 was a series of experiments at the Los Alamos Neutron Science Center (LANSCE) to measure energy deposition in the 5mm thick lithium drifted silicon detector by neutrons with an energy range from 20-600 MeV. The experiments were performed by integrating our 5mm silicon detector with the LANSCE time-of-flight neutron spectrometer on the 90 meter beam line to give simultaneous measurements of the incident neutron energy (LANSCE fission chamber) and energy deposited in our detector. Energy depositions of up to 150 MeV were seen from the up to 600 MeV incident neutrons in our 5mm detector. Complete analysis of the data from these experiments is on hold pending the resumption of funding in FY 2001. These high-energy neutron experiments complement and complete the measurement of neutron-silicon energy depositions from known monoenergetic neutrons begun at RARAF in1999. Together the LANSCE and RARAF neutron exposure data will enable us to develop a complete response function for the 5mm detector between neutron energies of 2 and 600 MeV. Inversion of this response function will allow us to calculate a most likely incident neutron energy spectrum, previously unknown, from a measured energy deposition spectrum.

The modeling component of this research program occurred on a continuous basis in FY 2000 until funds expired. We concentrated on modeling the high-energy channel from detailed cross sections of the basic neutron-silicon interactions using state-of-the-art computer codes. There are four reasons to develop this advanced modeling capability: 1) to assess the accuracy of the codes themselves to predict energy deposition in a silicon detector (by comparison with experimental data); 2) to use the codes in understanding the experimental results; 3) to determine whether the codes can be used to calculate the shielding and scattering effects of the instrument packaging and surrounding environment (structure or atmosphere); 4) to assess the ability of the codes to supplement the determination of the instrument response function at interpolated and extrapolated energies (since it is impractical to test at intervals of 10 MeV for the whole energy range). We have found that the GEANT4 code originally developed at CERN is the easiest to use, is maintained in a timely fashion by its developers and reproduces our RARAF (2-20 MeV) energy deposition data reasonably well.

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I. Research Plan Summary

A. Aims and Objectives from Original Proposal

The collision of high energy charged particles of extra-galactic, galactic, and solar origin with spacecraft structures in Earth orbit outside the atmosphere and in interplanetary travel beyond the Earth's magnetosphere create a number of secondary particles inside these structures that can produce a significant ionizing radiation environment. This radiation is a threat to long term inhabitants or travelers for space missions and produces an increased risk of cancer, DNA damage and central nervous system (CNS) damage. The primary, high energy cosmic rays and trapped protons collide with common spacecraft materials such as aluminum and silicon and create secondaries inside structures that are primarily protons and neutrons. Indeed, the effect of the structure is to convert primary protons into secondary neutrons. Charged protons are readily detected and instruments are already in existence for this task. Neutrons are electrically neutral and therefore much more difficult to detect. These neutrons are reported to contribute 30-60% of the dose inside space structures [Workshop98] and cannot be ignored. Currently there is no compact, portable and real time neutron detector instrumentation available for use inside spacecraft or on planetary surfaces where astronauts will live and work.

The aim of the original proposal was to support the NSBRI Radiation Effects Research Team (John Dicello, Team Leader) by supplying needed information on the neutron environment so that, combined with the work on damage to living organs, an accurate assessment of the increased risk of cancer or other radiation damage to astronauts could be made. Dose equivalence is calculated by dividing an environment spectrum into regions in energy with approximately constant damage equivalence factors, then summing the dose equivalent from each region. The inputs to this calculation are an environmental energy spectrum and the dose equivalent factors as a function of energy. The aim of our original proposal was to supply the means by which environment energy spectra can be measured for neutrons in a wide variety of applications.

The objectives of the original proposal were as follows:

1. Measure the energy spectrum of secondary neutrons generated by nuclear reactions from cosmic radiation hitting the shell of space structures.
2. Determine whether neutrons contribute 30-60% of the equivalent dose to spacecraft inhabitants.
3. Determine the characteristics, shape and slope of neutron spectra inside spacecraft or on planetary surfaces from 10 KeV to 500 MeV with an energy resolution of at most 10% for the various bins.
4. Design the instrument to be portable (brief case size) with a mass less than 10 kilograms so that it can monitor various locations within spacecraft and be transported onto planetary surfaces.
5. Actively record and process data and produce readouts of the desired parameter (neutron flux, dose or dose equivalent).

6. Include an alarm system to warn astronauts of high fluxes so that countermeasures such as seeking shelter or using an appropriate drug may be taken.

We proposed to design and build a portable, low power and robust neutron spectrometer that will measure the neutron spectrum from 20 KeV to 500 MeV with at least 10% energy resolution in the energy intervals. This instrument will be employed to monitor the existing neutron environment both inside spacecraft structures and on planetary surfaces to determine the safest living areas, warn of high fluxes associated with solar storms and to provide an accurate assessment of increased cancer risk and DNA damage to astronauts. Covering the large neutron energy range requires more than one detector system. The original proposal was for two detector systems: a Helium 3 proportional counter for low energy neutrons (10 KeV-5 MeV); and a silicon solid state detector for the several MeV to 500 MeV neutrons. The overlap in the energy ranges is by design to allow some cross calibration between the two detector systems in the 1-5 MeV interval.

The proposed final instrument, an engineering model, is intended to consist of two detector systems with associated electronics, read-outs and alarm packaged in a briefcase-sized configuration. It should be rack mountable or able to be transported to monitor the neutron environment inside space structures or on planetary surfaces. Using the proportional counter tube it will count neutrons less than 20 KeV in the region where thermal and epithermal neutrons are copiously available and where the damage factor is constant. Between 20 KeV and several MeV it will utilize the neutron capture reaction of the Helium 3 gas to produce charged secondary protons and tritons (^{3}H) which are easily detected. The cross section for the capture reaction falls sharply as MeV energies are approached, decreasing the efficiency of neutron detection significantly. For example, an 80% efficiency in the KeV energy range decreases to 0.1% around 1-2 MeV (See Figure A.1.). At several MeV, a solid state silicon detector is more efficient and will be responsible for monitoring the higher energy neutrons. The silicon solid state detector depends on both the elastic and inelastic collisions of neutrons with the silicon nuclei to provide spectra measurements. Secondary charged particles in the form of protons, alpha particles, spallation products or recoil nuclei are readily detected since an electron-hole pair is produced by ionization for each 3.64 eV of deposited energy. We also proposed to examine the options to boost the efficiency of the silicon detector by use of a moderator that converts neutrons to protons and/or a degrader to reduce secondary proton energy to a reasonable capture length.

In addition, it was recognized that an anti-coincidence shield/circuit would be necessary to discriminate against charged particles and that it would have to be quite efficient to keep the signal-to-noise ratio reasonable. Pulse height measurement electronics which are efficient and minimize noise were to be designed using our long experience with space instrumentation. Recent developments in electronics miniaturization would also be incorporated to insure portability and compactness. The availability of the design of a custom energy chip and other application specific integrated circuits makes significant miniaturization possible. Another technology driver is our success with chip on board

(COB) electronic packaging. Low power and robustness were to be allied goals in this development.

B. Modifications and Rationale for Modifications

The first modification was brought about by the unexpected availability of thick silicon detectors. The proposed instrument was to use a 500 μm silicon detector for measuring high energy neutrons. Thicker detectors (1-5 mm) are an order of magnitude more efficient than thin detectors for measuring neutron spectra as shown by early experiments with radioactive sources and basic nuclear physic modeling, but cost an order of magnitude more than thin detectors. As these thick detectors have become commercially available, we have incorporated them into the instrument at a higher than anticipated cost for the high energy sensor.

A second modification to the program came as a result of several reviews in which the selection of spacecraft materials for shielding and minimization of the neutron flux to astronauts has been emphasized. As a result, we incorporated requirements for ground-based shielding studies into the design of the prototype instrument and proposed a collaboration with researchers from NASA Langley and the University of California, Berkeley in response to NASA NRA 98-HEDS-05 to make measurements of secondary neutron spectra produced in energetic heavy ion interactions with candidate shielding materials. These measurements will be made in conjunction with secondary charged particle measurements made simultaneously by the other participants in the collaboration.

The third program modification came about as a result of discussions concerning the importance of thermal and epithermal neutrons to the overall dose equivalent of astronauts. Thermal and epithermal neutrons are abundantly produced in energetic particle interactions with materials, yet they do not contribute significantly to the dose received by an astronaut mainly due to their lack of penetrability into tissue (See Table A.2.). Since the flux of these very low energy neutrons obscures the more interesting neutrons with energy greater than 50 keV, the proposed concept of the prototype instrument was modified to include cadmium wrapping on the ^{3}He tube to absorb thermal and epithermal neutrons. This required some development on the part of the tube manufacturer to enclose the cadmium in a sealed, double-walled tube so that none of the volatile material is allowed to migrate to other spacecraft surfaces, preventing failure in other spacecraft systems.

The fourth modification came as a result of modeling the propagation of secondary protons in our proposed high energy channel design. This channel was originally conceived to be composed of two subsystems - a silicon solid-state detector for neutrons up to about 50 MeV and a proton telescope with a polyethylene converter for neutrons above 50 MeV. This design was proposed because the available data on neutron-silicon reaction cross-sections indicated that the efficiency of the silicon solid-state detector would decrease to an unacceptable level at higher neutron energies. This, however, proved not to be the case as further data became available; the total cross-section for neutron-silicon reactions remains relatively constant at 3-5% (refer to Figure A.19.) to

neutron energies as high as 150 MeV and beyond. In addition, extensive modeling of the polyethylene-telescope configuration showed that while the concept works, the efficiency of such an arrangement is approximately two orders of magnitude lower than the few per cent expected from a silicon detector alone. Therefore, the prototype instrument design was modified to include one or more thick silicon detectors in the high energy channel. This important result greatly simplified the high energy channel design.

The final program modification came as a result of the availability of high-altitude aircraft flight opportunities. NASA Dryden maintains a research facility in which instrumentation can be tested during flights of various aircraft at altitudes up to 70,000 ft. The average thickness of the International Space Station and the atmosphere on Mars are equivalent to the thickness of the Earth's atmosphere at about 70,000 ft, so high altitude tests were proposed to the NSBRI as a test of the prototype neutron spectrometer which could at the same time gather data of use to NASA in predicting astronaut neutron exposure. Adaptation of the prototype instrument to meet the requirements of the flight program required significant effort beyond the original concept for the neutron spectrometer. A self-contained power system (including a flight-qualified battery) and structure including acoustic dampening to allow the flight instrument to survive the vibration and thermal environments of the aircraft pod were added to the instrument. Environmental tests were required to demonstrate the mechanical integrity, safety and low-pressure/low-temperature operation before flight. None of these activities related to flight worthiness and autonomy were included in the original proposal for the neutron spectrometer.

C. Results and Accomplishments

1. Fiscal Year 1998

The main emphasis in the first year of the program was to purchase components for the initial concept testing of the neutron spectrometer and assemble them into preliminary versions of the low and high energy channels. For the low energy channel, we worked with LND, the selected tube manufacturer, to design a ^{3}He tube with optimum dimensions, wall material and gas characteristics for neutron detection. We built a multi-channel analyzer from this tube and commercial electronics to allow for testing of the tube, and then measured neutron spectra from sea-level atmospheric neutrons and from the ^{252}Cf source at the National Institute for Standards and Technology (NIST). Significant data produced in these tests are shown in Figure A.3. From these tests, we discovered that thermal and epithermal neutrons in ISS or planetary applications are expected to cause some degree of pulse pileup - an effect where two nearly simultaneous pulses appear as one larger pulse - in the gas tube. Pileup distorts measured spectra, and can be reduced to insignificant levels either by including pileup rejection in the electronics associated with the detector or by decreasing the intensity of the source causing the pileup. Thermal and epithermal neutrons are easily absorbed by cadmium, and so we chose to use a cadmium wrapped tube to eliminate the very low energy neutrons which cause pileup and reduce the signal-to-noise ratio of the tube.

The preliminary design of the high energy channel was developed in the first year of the program. Initially, four different silicon detector types were considered for the basis of high energy neutron measurements - dynamic and static random access memories, a PIN diode, and a 300 μm surface barrier detector. A multi-channel analysis system was built to test these detectors with various charged particle and neutron sources. In spite of encouraging work done by Clemson University in previous programs, exposure to alpha radiation indicated that the energy resolution of spectra measured by the dynamic and static RAMs was inadequate for neutron detection. A second set of tests with the remaining detectors and a thicker 500 μm silicon surface barrier detector purchased in the meantime with neutrons from a plutonium-beryllium source at Clemson University were carried out late in the first year. This data narrowed the choice of detector for the high energy channel to the thicker surface barrier detector. In addition, the effect of a polyethylene moderator on the energy deposition spectrum for neutrons was quantified as a function of polyethylene thickness. Data from these experiments is given in Appendix A, Figure A.4. The end result of this effort was to finalize the basic detection scheme for the high energy channel. At the end of the first fiscal year, we also purchased a 5 mm thick (the thickest commercially available) lithium-drifted silicon detector, as a potential candidate for the high energy detector, which was delivered in the second fiscal year of the program.

The original concept for the high energy channel was a stack of interleaved detectors and moderators, with the response of the detector composed of energy deposition spectra from secondary protons created in the moderator layers and from secondary charged particles created in neutron-silicon interactions in the silicon detectors themselves. The ability to invert the measured energy deposition spectra to get incident neutron spectrum depends heavily on models of the details of these interactions to provide a response function for the detector channel. The modeling effort in the first year concentrated on the secondary protons created in neutron-polyethylene interactions to better understand this contribution to the overall response function and to provide guidance for the design of the polyethylene/silicon layers in the stack or telescope concept of the high energy channel (i.e., layer thickness and separation, number of layers needed). Clemson University developed an analytic model of the neutron-polyethylene elastic interactions while APL began the development of a Monte Carlo neutron-silicon and proton-silicon interaction model. The data shown in Figure A.4 were successfully modeled as seen in Figure A.5 which clearly exhibits the optimum polyethylene thickness to maximize the count rate for the Clemson plutonium-beryllium radioactive source with a mean energy of 5 MeV.

2. Fiscal Year 1999

The first significant activity of FY 1999 was to expose two helium 3 gas tube proportional counter detector systems (one from LND and one from GE/Reuters-Stokes) and two bulk silicon detectors (one 500 microns in thickness and one 5 mm thick) to the NIST americium-beryllium radioactive neutron source which has a mean energy of about 5 MeV with a spectrum that extends up to 10 MeV at the high end. The 500 micron thick

silicon detector showed a response quite similar to results previously obtained at Clemson in April 1998 (Figure A.4). Data from these experiments are given in Appendix A; Figure A.6 shows the greatly enhanced response of the 5mm detector particularly in the 0.5-4 MeV energy range corresponding to the difference in thickness between the two types of silicon detectors. Much of the contribution in the 0.5-2 MeV range was deduced to be from background gamma rays. We did not perform a separate background measurement at NIST since the neutron source was always present in the room either inside or outside its water shield. We detected some low number of events with the 5mm detector even with the source inside its shielding container, so the bucket of water was far from a perfect shield. However, this data was sufficient to make several design decisions for the integrated spectrometer.

During November 1998, several significant decisions were made as an outcome of the October 1998 NIST tests and some lengthy discussions. Dr. Fainchtein decided to use the GE/Reuters-Stokes helium 3 detector tube because of the greater purity of the helium 3 gas inside the tube and the ability of the manufacturer to produce a tube with an inner, but isolated cylindrical cadmium partition that will remove the high fluxes of thermal and epithermal neutrons from the gas detector and enable us to concentrate on the neutrons above 10 KeV. We also decided that we would use a scintillator cup with PIN photodiode detectors for the detection and discrimination against charged particles, primary and secondary protons and cosmic rays which will pass through the neutron silicon detectors depositing energy as they do so. Since the charged particles will also deposit charge in the CsI scintillator before entering the silicon detector(s), but electrically neutral neutrons will not, any light detection by the PIN photodiode system will veto subsequent near time detection by the silicon detectors. An ancillary activity was visiting and evaluating several monoenergetic neutron facilities for future more precise work on detection efficiency and energy resolution for the preferred helium 3 tube and the 5mm thick silicon detector. We chose Columbia University's RARAF which can supply monoenergetic neutron beams from 0.5-3 MeV, at 5.89 MeV and at 14 MeV using proton-lithium, deuteron-deuterium and deuteron-tritium reactions respectively. The last of these reactions is truly impressive because a 0.60 MeV deuteron is accelerated by the Van de Graaff, strikes a tritium target and produces 14 MeV neutrons at an angle of 100 degrees. The source of this energy amplification is the binding energy of the tritium atom released in the fusion event.

During February 1999, we created the block diagram of the integrated neutron spectrometer system concept. The concept was presented for the first time at the visit of the NSBRI External Advisory Council to APL on February 23, 1999, and is shown in Figure A.8. The three detector systems for low (< 3 MeV), medium (1-20 MeV) and high (>20 MeV) energy neutrons combined with their individual analog electronics are interfaced with common power conditioning and data processing units. A single cadmium shielded tube is the most efficient configuration. We estimated that we were well below our original mass objective of 10 kilograms, while the 4 watt power limit may be slightly exceeded depending on the amount of DC/DC conversion necessary to produce the high voltage for the ^{3}He tube and 5 mm bulk silicon detectors. Based on more recent work at Columbia University and extensive modeling of neutron-silicon interactions, this block

diagram was greatly simplified by combining the medium energy and high energy channels into one high energy channel which uses one or more thick silicon detectors. For comparison Figure A.9 shows the more compact block diagram of the MANES instrument proposed for the Mars 2003 Lander.

In the second week of February we carried out our first round of experiments at Columbia University RARAF. The response of the GE/Reuters-Stokes ^{3}He tube to neutron energies of 0.50, 0.84, 1.47, 1.97 and 2.46 MeV was "textbook". The results shown in Figure A.10 for 2.46 MeV neutrons are actually the detailed outcome of three visits to RARAF in February, June and October 1999. The 5mm bulk silicon detector was exposed at three neutron energies, 2.46, 5.89 and 14 MeV, and showed an efficiency of detection of ~1% at the lowest energy and 4-5% at the two higher energies. The efficiencies demonstrated by the 5mm detector were a pleasant surprise. In addition, considerable structure was seen for high energy deposition events in the 5mm detector exposed to 14 MeV neutrons (see Figure A.11). We believe these peaked structures are due to the presence of the appropriate inelastic channels that open and occur as the energy of the neutron-silicon collision increases. Later analysis from neutron-silicon modeling indicated that a significant gamma ray flux may exist during the times when the RARAF beam is on, even though we detected only low gamma flux in the room with the beam off. The effect of this gamma flux on our measurements was to increase the low energy deposition portion of the spectra obtained with the silicon detectors, which can be removed by background subtraction, by the use of anti-coincidence detectors to veto counts from gamma rays, or by increasing the low energy threshold of the spectra obtained from the silicon detectors. In the final neutron spectrometer, a combination of all three gamma contamination reduction measurements will be used.

We completed a second set of neutron exposures at RARAF in June 1999. The main focus of the exposures was to test the thick silicon detectors with a mono-energetic neutron beam at higher energies than previously examined. This data was the baseline verification to evaluate the accuracy of neutron-silicon interaction models. The data consists of energy deposition spectra for 5.90, 14.0, 16.3, and 18.5 MeV incident neutron energies and is shown in normalized form in Figure A.20. The experimental detection efficiencies demonstrated by the 5 mm detector are compared to existing NASA and DOE nuclear cross section models in Figure A.19. In addition, we repeated exposures of the GE/Reuters-Stokes ^{3}He tube with cadmium wrapping to examine the effects of the cadmium on spectra obtained from the tube.

Early in FY 1999, the Clemson model of the neutron-silicon elastic interaction, begun in FY 1998, was completed. This task involved understanding the Clemson formalism and analytical results, checking the results for accuracy and implementing them in a usable computer language. The modeling of the neutron-silicon elastic reaction is one of the key physics of collisions principles necessary to understanding the energy deposition events in a bulk silicon detector. We had received our first 5mm thick bulk silicon detector at the end of September 1998 and used the modeling to simulate the low energy deposition events that we expected to see experimentally when the 5mm and 500 micron thick detectors were exposed to the NIST americium-beryllium neutron source. We also began

the phenomenological model for the silicon detectors using the mono-energetic exposures at Columbia's RARAF. We collected secondary particle spectra for several energies and began the process of deriving response functions for the 5 mm detector from this data. From these energy deposition spectra we will be able to calculate the statistically most likely incident neutron spectra from mixed energy neutron radiation.

In addition, James Kinnison completed the modeling of the neutron-polyethylene moderator interactions for the high energy detector stack which was begun in December 1998. The basics of the neutron elastic scattering from the hydrogen atoms in the polyethylene material were readily completed. The simulations run to determine the optimal moderator thickness were executed in January 1999. This software module takes the incoming neutrons in the 50-500 MeV energy range, calculates how many interact in a given volume of polyethylene, determines the energy and direction of the secondary protons produced and yields, as an output, how many of these protons emerge from the moderator's lower surface and enter the stack of silicon detectors below the moderator where these charged protons are detected with ~100% efficiency. The results showed that a polyethylene thickness of 1 centimeter optimized the secondary proton production efficiency throughout the range. This efficiency is greatly enhanced for the lower energies in the range (50-200 MeV) where the mean interaction length of the neutron is less than or on the order of the thickness of the moderator. As the neutron energy becomes very large, the efficiency response curve tends to flatten for all thicknesses modeled since the mean free path is longer than the polyethylene thickness. The result of this phase of the modeling effort are given in Figure A.7.

The second major modeling task undertaken in 1st quarter CY99 was the interaction of the secondary protons produced in the moderator with both a thin 300 micron silicon transmission detector and a 500 micron standard surface barrier detector. These two detectors separated by an appropriate thickness of air and/or metallic degrader together with the polyethylene moderator conceptually compose the high energy neutron detector stack or telescope system. Dr. David Roth completed this software module in February 1999 and began to integrate it with neutron-polyethylene module in March. Executing the hundreds of runs necessary to track at least 10,000 protons produced by neutrons of 50-500 MeV initial energies and at several detector separations between 0 and 100mm with and without the presence of a tantalum degrader occupied three different computers and took several weeks. Results are shown in Figure A.12. Thus, the theoretical model for the high energy recoil proton telescope is a strictly Monte Carlo simulation of proton production in the moderator, as well as propagation and detection in the layers of the stack. For each input neutron, the model first randomly calculates the secondary proton energy, if any, in the moderator, then propagates that proton outside the moderator and determines the energy deposited by that proton in each detector through which it passes. From this information we can predict the resultant secondary proton spectrum in the detector stack for any given neutron spectrum. This model gave the first indication that thick silicon detectors are more efficient than the proton telescope for high energy neutron detection , and that great simplification could be achieved by combining the high and medium energy channels into one channel which spans the energy range of both

previous channels. Additional evidence was supplied by the detection efficiency achieved by the 5mm thick bulk silicon detector at Columbia RARAF in June 1999 (Figure A.19).

The final noteworthy event from the December 1998-January 1999 time period was obtaining both a commitment from NASA Dryden to fly a version of our neutron spectrometer on F-18 aircraft during the summer of 1999 and the additional funds from NSBRI to meet the flight opportunities. The additional funding enabled us to begin designing and equipping the aircraft flight package. Several meetings and extended discussions took place on the topic. We were able to make use of much of the equipment already purchased at APL for the aircraft flight instrument. However, the system assembled for the aircraft flight differed from the prototype instrument in that an external battery for power during flight needed to be added, and the hardware package needed to withstand flight vibration and temperature environments fo the high altitude aircraft. These were accomplished in the third year of the program with the help of Stephen Corda at NASA/Dryden.

3. Fiscal Year 2000 (Activity since the May 1999 Annual Report)

(a) Engineering Prototype Hardware and Aircraft Flights

The design, parts selection and parts procurement for the NSBRI engineering prototype neutron spectrometer for F15/18 aircraft flights was carried out between April and August 1999. A battery of the correct size, capacity and safety was one of the major considerations. The transition from design to fabrication was interrupted by the proposal to fly a version of our neutron spectrometer on the Mars 2003 Lander in response to NASA AO 99-HEDS-01. The proposal effort took a significant amount of time in July and August 1999.

After we finished the Mars 2003 Lander proposal, we began fabrication of the engineering prototype in September 1999. By early September John Goldsten had integrated a helium 3 tube, a preamp box, a new analog electronics board and a newly designed digital processing board on the bench for performance testing. We decided to use wire wrap boards for fabrication to minimize the costs. Dave Roth supplied the high-energy detector and associated electronics. With some technician and APL shop help, the battery, high voltage supplies, DC/DC converters and pressure sensor were combined with the detector modules. The packaging case and base plate to tie the spectrometer to the aircraft pod were completed during the second half of October 1999. Additional calibration tests for rise time discrimination with the helium gas tube were performed at Columbia RARAF at the end of October.

To begin qualification for the aircraft flights we asked one of APL's mechanical analysts to evaluate our design versus the F15/18 vibration specifications supplied by NASA Dryden. The analyst recommended that we add brackets, bosses, staking and taping to insure survival in what he considered a demanding vibration environment. He also helped design an acoustic cover to reduce the microphonic noise seen by the gas tube due to the simulated vibration. These improvements were in the process of being accomplished

during November 1999 when our selection for the Mars 2003 Lander diverted our work for a second time.

As described in the section on MANES most of December 1999 and January 2000 were spent answering action items with respect to Mars 2003 Lander interfaces and other accommodation issues. Of course, this effort proved to be futile since the original 03 Lander mission has been canceled.

We returned to the engineering prototype hardware again in February 2000 after the recommended mechanical enhancements had been done. The sine sweep and random vibration qualification was successfully completed on February 15-16, 2000 with little difficulty. Encouraged by the success in mechanical test we then continued with cold temperature and high altitude testing the week of February 21-25, 2000. We simulated temperature of –65 degrees F and an altitude of 45,000 feet (~1-2 psi) in the chamber allowing some margin on each limit since the aircraft were to fly at 40,000 feet. Cold temperature performance was superb, but we did experience corona and high voltage break down in the instrument.

Using a Test, Analyze and Fix (TAF) approach, we found a connection in a high voltage path that had escaped potting originally. After we potted the joint we redid the altitude test. We were successful in operating the instrument for about six hours at the pressure simulating 45,000 feet and judged that we were qualified for flight. The subsequent corona and break down damage that we experienced in flight at 39,000 feet may be due to incorrect chamber calibration in view of the small margin we were using or the variability in air pressure over the dry California desert over small increments in altitude.

We delivered the neutron spectrometer prototype (Figures A.13 and A.14) to NASA Dryden on March 23, 2000 and after fit checks, review and approval were scheduled for flight on an F15 the week of April 24-28, 2000. Both John Goldsten and Dave Roth were present for the flights that were actually carried out on April 25-26 (See Figures A.15 and A.16). We have data on the aircraft ascent up to 39,000 feet, shown in Figure A.17, at which height we experienced corona, break down and instrument failure. The Low Energy Spectrometer (LES) experienced a blown JFET which was the same problem seen in the altitude qualification chamber; the High Energy Spectrometer (HES) experienced both a blown pre-amp and shaping amp.

We are rebuilding the high voltage sections of the instrument. For the LES we are redesigning the high voltage section so that all components will fit in a small box for the gas tube. This box will be potted and mounted directly to the tube. Connectors will be filled with high viscosity silicone grease to eliminate any voids. For the HES we have fabricated a solid box with a grooved lid and a gasket to form a small pressure vessel that will maintain the pressure for the high-energy silicon detector at one atmosphere. Resumption of activity will occur with new funds in FY 2001.

(b) MANES (MArtian Neutron Energy Spectrometer)

Our proposal titled "MArtian Neutron Energy Spectrometer (MANES) for the Mars 2003 Lander Mission," submitted in August 1999 in response to NASA AO 99-HEDS-01, was selected for a stage of further definition as a potential instrument on the Mars 2003 Lander in November 1999. Due to subsequent cancellation of the Mars 2003 "large" lander we are now on hold until a decision is made about a Mars 2005 Lander which should occur this fall, possibly late September. We did receive an extended definition phase grant of $123,000 to study various instrument configuration options as contingencies for future mission concepts from NASA Johnson for the period of February 15, 2000 to August 11, 2000. A cross sectional view of the MANES instrument as proposed is shown in Figure A.18. The block diagram is shown in Figure A.9.

Immediately following our selection in November 1999 we had to prepare for and attend a Mars 2003 Lander payload accommodation meeting in Pasadena on December 7-9, 1999. Action items on instrument mass, volume, power consumption including survival heat and operating modes were due and answered by January 10, 2000. This flurry of activity again caused an interruption in the progress on the aircraft flight hardware qualification.

Due to the failures of the September and December 1999 Mars' missions the whole Mars Surveyor program was put on hold at the end of January, 2000. Subsequently, NASA decided to fly only an Orbiter in 2001 (April 2000 decision) and two smaller Athena Rover/Landers in landing bags in 2003 (July 2000 decision). The first decision and anticipation of it by NASA JSC resulted in a request to consider the option of combining Dr. Gautam Badhwar's Mars 2001 Lander MARIE instrument with our MANES instrument for a possible large Mars 2003 Lander. This combination was called MARINES. A technical interchange meeting was held at JSC on March 1-2, 2000 and a formal presentation was made on both MANES and MARINES at NASA Headquarters on March 29, 2000. This effort necessitated not only the summary of past work on MANES but also new design concepts for MARINES.

The July 2000 decision to fly the two Athena Rovers in 2003 has resulted in any future JSC funding being put on hold for MANES until NASA makes a decision about the Mars 2005 mission. JSC's justification for this decision is that starting sometime in FY 01 is early enough to develop, build and qualify an instrument for 2005. Between the April and July decision dates on the Mars Surveyor program we submitted (5/30/00) a revised proposal and statement of work for an extended definition phase for MANES for an anticipated Mars 2005 Lander at the request of NASA. We asked for $575,000 for the sixteen-month period from June 1, 2000 to September 30, 2001. It is this funding amount that is now in hold.

Dr. Richard Maurer attended the "Concepts and Approaches for the Robotic Exploration of Mars Workshop" in Houston on July 18-20, 2000. The results of NASA requests for input from the universities, research institutions, NASA centers and industry will be summarized in a white paper. The initial draft is to be circulated for comment by October

1, 2000 with the final version to be published by Thanksgiving. It is noteworthy that the JSC/JPL/Ames HEDS program office presented a concept of MARSLAB which would be a HEDS only lander possibly using the existing but now canceled Mars 2001 Lander as a starting platform.

(c) NASA Materials Science Grant

Our proposal titled "Development of a Neutron Spectrometer to Assess Biological Radiation Damage Behind Spacecraft Materials," submitted in March 1999 in response to NASA NRA 98-HEDS-05, was selected for funding in January 2000. Due to the delayed award date for FY 00 funding from NASA Marshall for only $45,000 was received for the period from May 1, 2000 to November 30, 2000. The funding will continue for three additional years from December 1, 2000 to November 30, 2003 at $90,000 per year for a total of $315,000. Since the amount awarded was slightly less than the amount requested ($100,000 per year and a total of $400,000) NASA Marshall required us to submit a recosted proposal in March 2000 before authorization could occur.

Our primary responsibility under this grant is to support the Lawrence Berkeley Laboratory (LBL) personnel in the evaluation of spacecraft structural and shielding materials by supplying a version of the neutron spectrometer suitable for accelerator tests. Due to downtime, repairs and refurbishment of various accelerator facilities in FY 2000, the first time we are scheduled to support the LBL experiments is January 8, 2001 at Brookhaven National Laboratory (BNL). These experiments will collide high-energy heavy ion beams with standard and novel spacecraft materials and our spectrometer will measure the neutron energy spectrum produced as a result of these collisions. We already have the necessary electronics and neutron detector for these tests and need only obtain the charged particle discrimination medium/detector configuration to be employed. The BNL experiments will take place after we receive our second increment of funding from Marshall on December 1, 2000.

A secondary responsibility for this grant is to develop the modeling effort in a manner that is useful for materials science experiments as well as for assessment of astronaut biological radiation risk. Our success using the GEANT4 Monte Carlo code in modeling the neutron silicon interactions will supplement the current NASA modeling efforts which employ deterministic Boltzmann transport and FLUKA Monte Carlo approaches.

We participated in the NASA Microgravity Materials Science Conference, June 6-8, 2000 in Huntsville, AL by presenting a poster paper on our neutron spectrometer concept for the accelerator experiments. We were prevented from reaching the Shielding Materials Workshop in Berkeley, CA, August 8-9, by the United Airlines pilot slowdown. However, we have submitted our comments on the workshop minutes to Dr. James Adams of Marshall.

(d) Energy Deposition Measurements

In October 1999, a third set of experiments were completed at RARAF using the GE/Reuters-Stokes ^{3}He tube to investigate the use of rise-time discrimination to separate pulses from capture events from elastic collisions in the tube energy deposition spectrum. When these effects are separated, the process of calculating an incident neutron spectrum from the separate energy deposition spectra is simplified, and so can give lower uncertainty in the calculated neutron spectrum than for spectra in which the two event types are mixed. We were able to use rise-time discrimination to measure separate capture and elastic spectra. The results of these exposures are given in Figure A.10. However, further analysis indicated that the usefulness of rise-time discrimination may be limited in field applications by the presence of background charged particles, which can produce pulses which look like those produced in elastic interactions. Further experimentation is necessary here.

In August 2000 we completed a series of experiments at the Los Alamos Neutron Science Center (LANSCE) to measure energy deposition by neutrons with an energy range from 20 - 600 MeV. This was accomplished by integrating our 5 mm silicon detector with the LANSCE time-of-flight neutron spectrometer on the 90 m beam line to give simultaneous measurement of the incident neutron energy and deposited energy in our detector. This experiment completes the measurement of neutron-silicon energy deposition begun at RARAF the previous year, and combined with the results of those exposures will yield a complete response function for the thick silicon detector to allow calculation of a most likely incident neutron spectrum from a measured energy deposition spectrum. In addition, the effect of polyethylene moderation on the measured energy deposition spectrum was examined for several moderator thicknesses used to simulate human body tissue, and the effect of the aluminum housing around the silicon detector was examined. Preliminary raw data from these experiments is given in Figure A.21.

(e) Neutron-Silicon Modeling

In fiscal year 2000, we concentrated on modeling the response of the high energy channel from detailed cross-sections of the basic neutron-silicon interactions using state-of-the-art computer codes. The purpose for developing models was to assess the accuracy of these codes for neutron-silicon interactions, to use these codes to understand the results of our energy deposition measurements, to determine whether these codes can be used to calculate the effects of packaging and instrument surroundings on the incident neutron spectrum, and to assess the ability of the codes to supplement the experimental determination of the instrument response function. We first developed a model of the high energy channel in MCNPX, a combination of MCNP which is widely used in the nuclear engineering and LAHET which is often used in accelerator design and other high energy physics calculations. The model did not reproduce the energy deposition spectra measured at RARAF. Further investigation showed that neutron calculations in MCNPX are performed in such a way as to preclude use of this code for energy deposition calculations without a major restructuring of the code. In the meantime, GEANT4, a code

library widely used in high energy detector design and simulation produced at CERN, was released. Our first model using GEANT4 uncovered several problems with the library, which were quickly addressed by the developers at CERN. The latest version of our model using GEANT4 reproduces the energy deposition spectra measured at RARAF reasonably well, although discrepancies for the highest energy depositions remain to be resolved. One interesting result of this model is a discrepancy at low energies indicates we may not have adequately accounted for gamma production during exposures at RARAF by measuring the background spectrum when the beam is off. While this does not invalidate those measurements, it does indicate the need for accurate background determination and/or rejection even in controlled laboratory exposures. Results from the modeling, including comparison with RARAF exposure, are given in Figure A.22.

II. Implications for Future Research

A. Detector Considerations

Two gas tube proportional counters are necessary if one wants to count the thermal and epithermal neutrons in addition to measuring the neutron energy spectrum starting at a minimum energy of 20 KeV. One tube has a cadmium inner wall and measures the low energy spectrum without the thermal/epithermal neutrons while the second has a plain aluminum or stainless steel wall and includes the thermal/epithermal neutrons in its counts. The difference between the counts between the shielded and unshielded tubes is due just to the thermal/epithermal component. However, as indicated by Dr. John Dicello, since only neutrons above 20 KeV are of interest with respect to radiation damage to tissue and risk of carcinogenesis, then a single cadmium shielded helium gas tube will be sufficient.

The 5mm thick lithium drifted silicon solid state detector has proved to be 3-5% efficient in detecting neutrons between 2.46 and 18.5 MeV at the Columbia University RARAF (Radiological Research accelerator Facility). Since computer simulations of a stack of thinner silicon detectors in a telescope arrangement predict detection efficiencies on the order of $5X10^{-4}$ at neutron energies of 50 MeV and above, we are concentrating on extending the applicable energy range of the 5mm bulk detector to hundreds of MeV since it is demonstrating two orders of magnitude better efficiency. We have already taken data up to neutron energies of 600 MeV at the Los Alamos Neutron Science Center (LANSCE) in August 2000. This data is currently under detailed analysis, but we do know that we observed energy depositions of a maximum of 150 MeV from these experiments using a single 5mm detector. The MANES proposal was to combine or gang two 7mm (the thickest any manufacturer will promise to deliver) thick lithium drifted silicon detectors together to give us 1.4 cm of detector thickness. For an International Space Station (ISS) application in which we are allowed more volume and mass we are considering several 5-7mm thick detectors in a small array to increase the neutron count rate.

B. Ancillary Materials

Since we have abandoned the telescope concept due to its very low detection efficiency, we are presently not planning on the use of any significant moderator or degrader material unless directionality of the neutrons is important as is the case on Mars. For directionality MANES will use a yet to be determined thickness of polyethylene sandwiched between two proportional gas tube counters to eliminate albedo low energy neutrons from the detector looking at the atmosphere and vice versa. We will place a thin layer of boron in the middle of the polyethylene thickness to absorb moderated neutrons and prevent them from propagating to the more distant detector.

C. Anti-coincidence shield/circuit design

The bulk silicon detector(s) will be surrounded by a CsI scintillator to record and veto the signals produced by charged particles that are not of interest for the neutron spectrometer but deposit energy in the silicon detector. During the summer of 2000 we have been evaluating the performance of large area (10X10 mm-28X28 mm) PIN photodiodes and obtained very favorable results with respect to detection efficiency and energy resolution when compared to bulkier photomultiplier tubes (PMT). We believe that PIN diodes can be used successfully up to an area of 40X40 mm if good energy resolution is necessary and for even larger areas if just the veto capability is used.

D. Electronics Miniaturization

The best example of this progress is to compare the pictures of the existing engineering and aircraft flight prototype, Figures A.13 and A.14, to the cross section view of the MANES proposal concept, Figure A.18. The mass of the aircraft flight instrument is 24.5 kg; the estimated mass of the MANES instrument is 4.9 kg.

E. Spectrometer Calibration

Essentially all final testing, evaluation and calibration are and will continue to be done using mono-energetic accelerator beams. We do employ a Californium radioactive source for preliminary low energy detector noise determination and rise time measurements on the bench in the laboratory. When the charged particle anti-coincidence scintillator shield or enclosure is integrated into the instrument, we will also use proton and heavy ion beams to check the charged particle rejection efficiency and the usefulness of this information with respect to measuring charged particle spectra.

F. Modeling

The modeling effort is being iterated with the accelerator experiments to develop an instrument transfer function. At present we plan to continue with the use and development of GEANT4. We get helpful responses to questions and problems from CERN via email. GEANT4 is the code chosen for use by ESA for materials radiation shielding applications, is of interest to NASA for the same reason and is also being used

by NASA scientific instrument designers to calculate induced radioactivity in spacecraft which influences the environmental background.

G. Biomedical Research

The biomedical research that benefits from our developments is that on the risk of radiation exposure for long term spacecraft inhabitants and travelers. An enhanced instrument that could provide additional benefit is one that would combine the neutron spectrometer with a charged particle spectrometer for one unified radiation monitor. A potential spin-off would be a portable and low power neutron dosimeter for DOE applications. A second is to use the low energy neutron detector for the indirect remote detection of water, a development that is already under way in APL's MESSENGER mission to Mercury scheduled for launch in 2004. Third, Dr. Raul Fainchtein has a NASA PIDDUP grant for using a radioactive source to produce neutrons for gamma ray spectroscopy of planetary surfaces from a lander. Fourth, we have already mentioned the funding that we have received from NASA Marshall to use a version of our neutron spectrometer to investigate the effectiveness of spacecraft shielding materials. An interesting part of this study is the capacity of the spacecraft material to not produce copious numbers of secondary neutrons while providing reasonable protection against primary charged particles. Finally, several aircraft and balloon flights would yield interesting data on atmospheric neutrons near Solar Maximum at the beginning of the 21st century.

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Appendix A

A. Low Energy Detector Efficiency

The chief feature of energy deposition in the ^3He tube is the capture reaction in which neutrons are fully absorbed in the proportional gas with an energy release of

$$^3He + {}^1n \rightarrow {}^3H + {}^1p \quad 0.764 \text{ MeV}.$$

We used this feature to calibrate the energy scale of the detector under the assumption that background neutrons captured without an excitation source present are thermal and/or epithermal. The energy of the thermal and epithermal neutrons that arrive at the detector is very small compared with the 0.764 MeV energy released by the reaction and can therefore be ignored. These conditions provide two parameters to linearize the MCA channel to energy conversion scale.

$$\text{Energy (eV)} = (\text{Channel} \times 0.764 \times 10^6 \text{ (eV)} / \text{Channel}_{peak}) - 0.764 \times 10^6 \text{ (eV)}$$

In addition to the energy calibration we have to take into consideration the detector efficiency. This is given as

$$\text{Efficiency} = 1 - \exp(-\sigma N x_m)$$

where σ is the total cross section of ^3He to neutrons. This includes contributions from the ^3He(n, p) capture reaction, the elastic ^3He recoil collision reaction and the ^3He(n, d) reaction. However, this last reaction does not have a contribution to the cross section for energies below 3.5 MeV. The cross-section as a function of energy is given in Figure A.1. The decrease in the capture cross-section causes the detector efficiency to drop to 0.1% or less of its maximum value above 1 MeV.

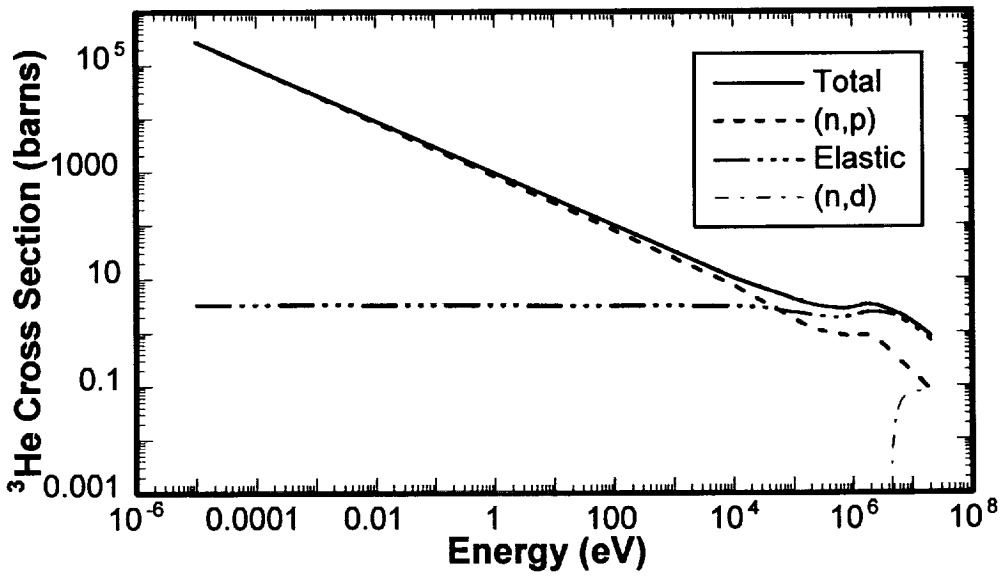


Figure A.1 3He neutron cross-section as a function of energy for efficiency calculations.

B. Dose Equivalent Weighting Factors

Radiation Component	Weighting Factor ICRP	Weighting Factor NCRP
X-rays, gamma rays, electrons, positrons, muons	1	1
Neutrons with energy of < 10 KeV	5	5
10-100 KeV	10	10
> 100 KeV → 2 MeV	20	20
> 2 MeV → 20 MeV	10	10
> 20 MeV	5	5
Proton with energy of > 2 MeV	5	2
Alpha particles, fission fragments and nonrelativistic heavy nuclei	20	20

Table A.2. Recently recommended radiation weighting factors. Ref.: John W. Wilson et. al, Radiation Safety Aspects of Commercial High Speed Flight Transportation, NASA Technical Paper 3524, May 1995. Neutrons with energy less than 10 keV can be ignored since the relative contribution to the dose equivalent is negligible due to their lack of penetrability. Neutrons above 20 MeV can not be ignored enev though they have the same weighting factor as thermal/epithermal neutrons since they penetrate deeply within the body.

C. 3He Measurements with NIST Source

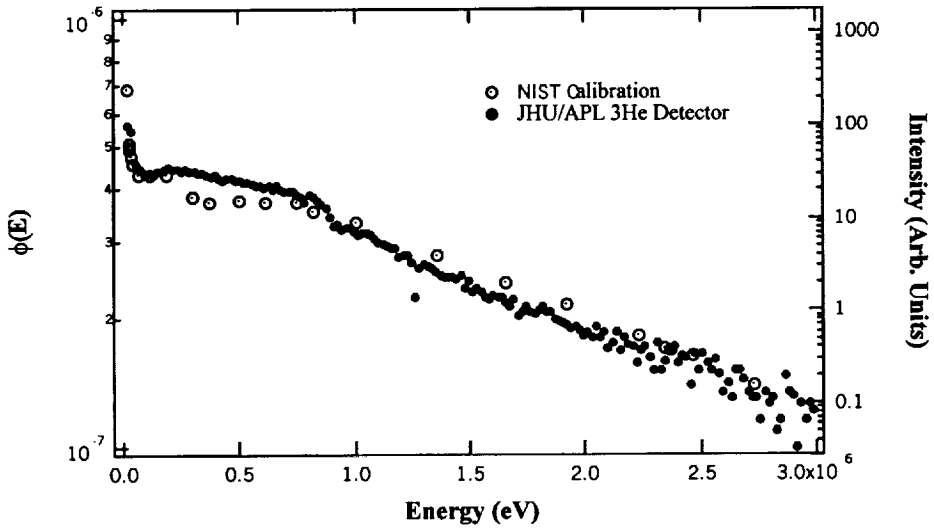


Figure A.3. Comparison between the NIST calibrated spectrum for the ^{252}Cf source and the spectrum obtained from the ^{3}He proportional counter.

D. Clemson Moderator Experiments

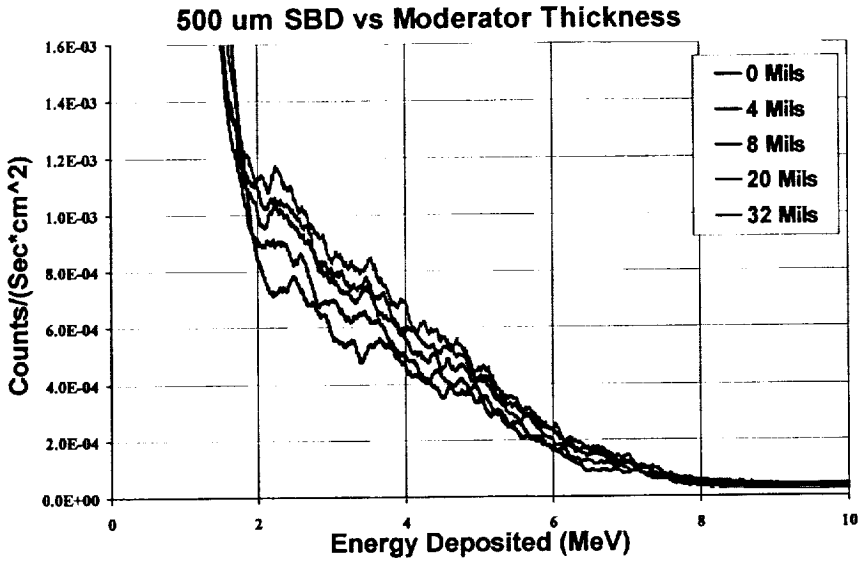


Figure A.4. 500 um SBD versus polyethylene moderator thickness experimental data from a Pu Be radioactive source at Clemson University. Note the increasing count rate for thicknesses up through 20 mils but decreasing count rate at 32 mils.

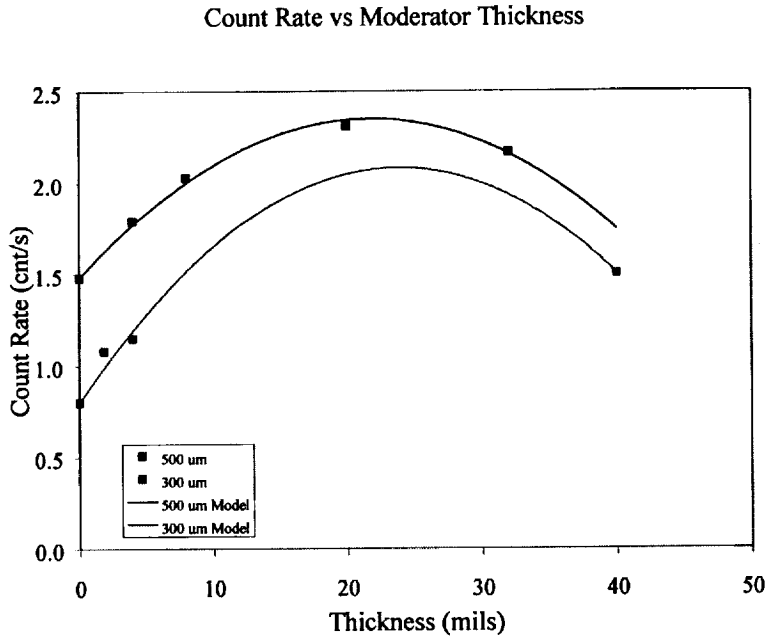


Figure A.5. Count rate versus polyethylene moderator thickness for energy depositions greater than 2 MeV in the detector due to the ^{239}PuBe neutron source. Comparing the model to the data showed that proton production is a complex function of moderator thickness and that a given moderator thickness, in this case 22 mils, is optimum for a given neutron energy, in this case a mean of 5 MeV

E. Silicon Detector Experiments at NIST

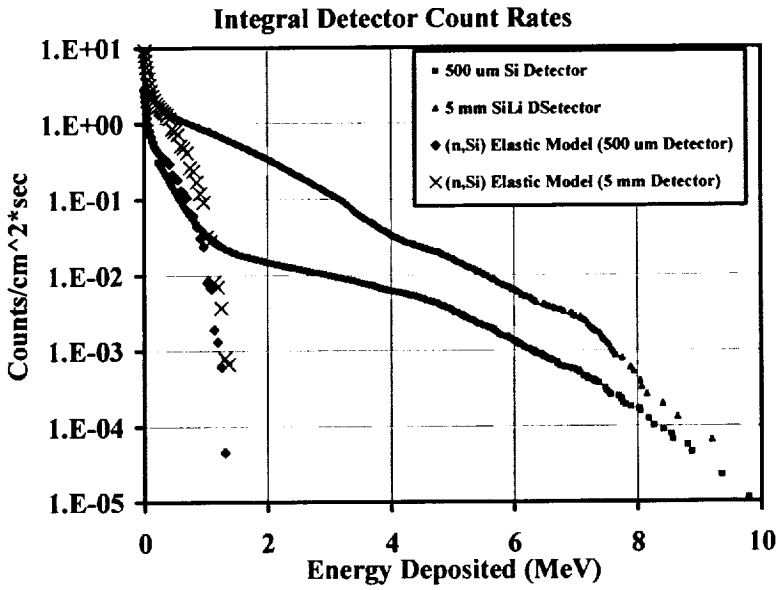


Figure A. 6. Integral deposited energy spectra comparison of the 500 um Surface Barrier Detector and the 5 mm lithium drifted silicon detector exposures at NIST. The 5mm detector demonstrates as much as an order of magnitude higher count rate for the 5 MeV mean energy Am Be source. The results of the neutron-Si elastic scattering model for the NIST source are also shown for each detector.

F. High Energy Neutron-Moderator Model

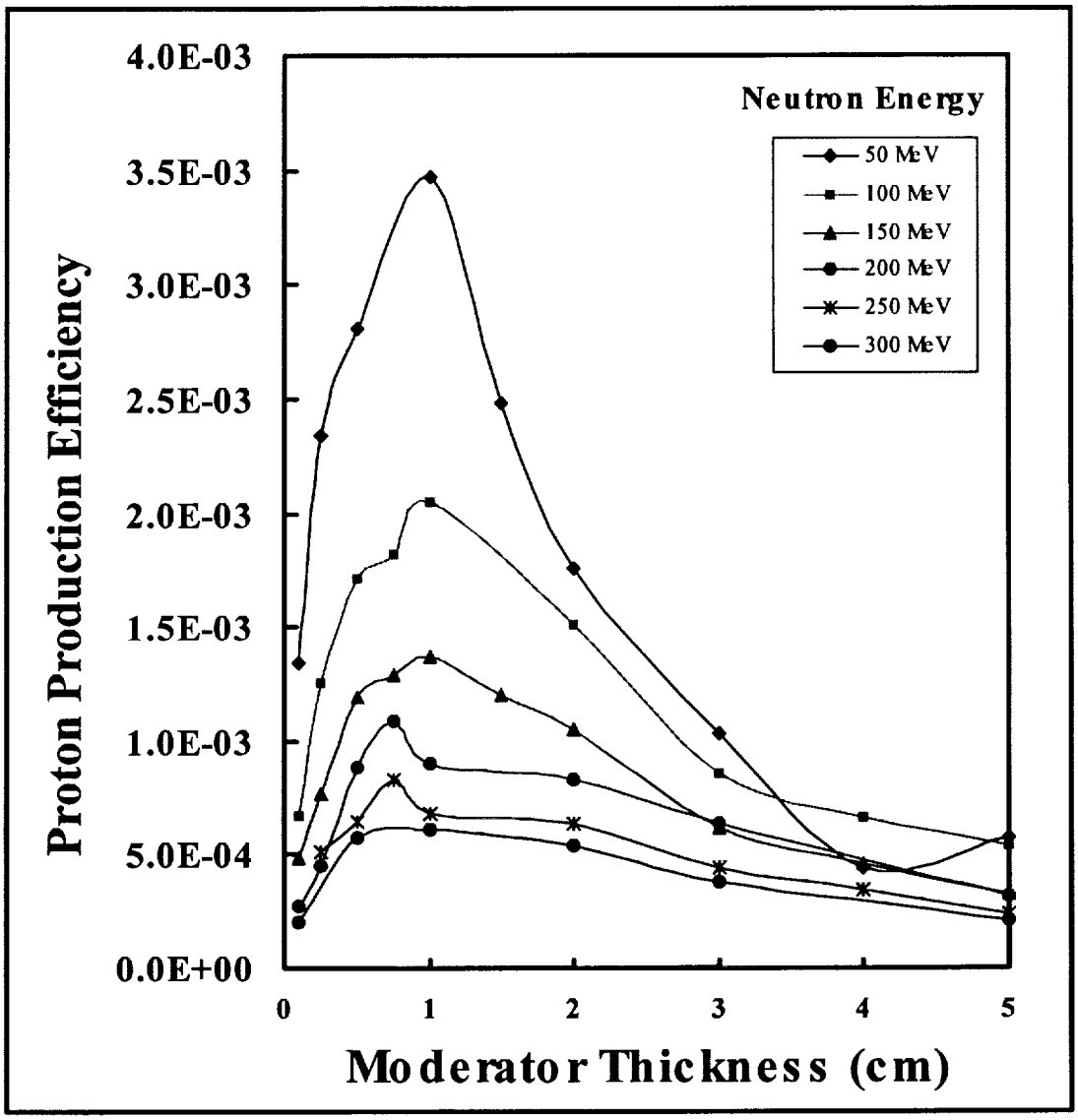


Figure A.7. Secondary proton production efficiency versus moderator thickness for high energy neutrons in the range 50-300 MeV. The results are based on Kinnison's neutron-polyethylene elastic reaction model. The results show that as very high neutron energies are approached, the proton production efficiency of the moderator decrease to 0.1% or less for all practical thicknessess. These results imply that any proton recoil telescope detection system will be similarly limited.

G. First Spectrometer Block Diagram

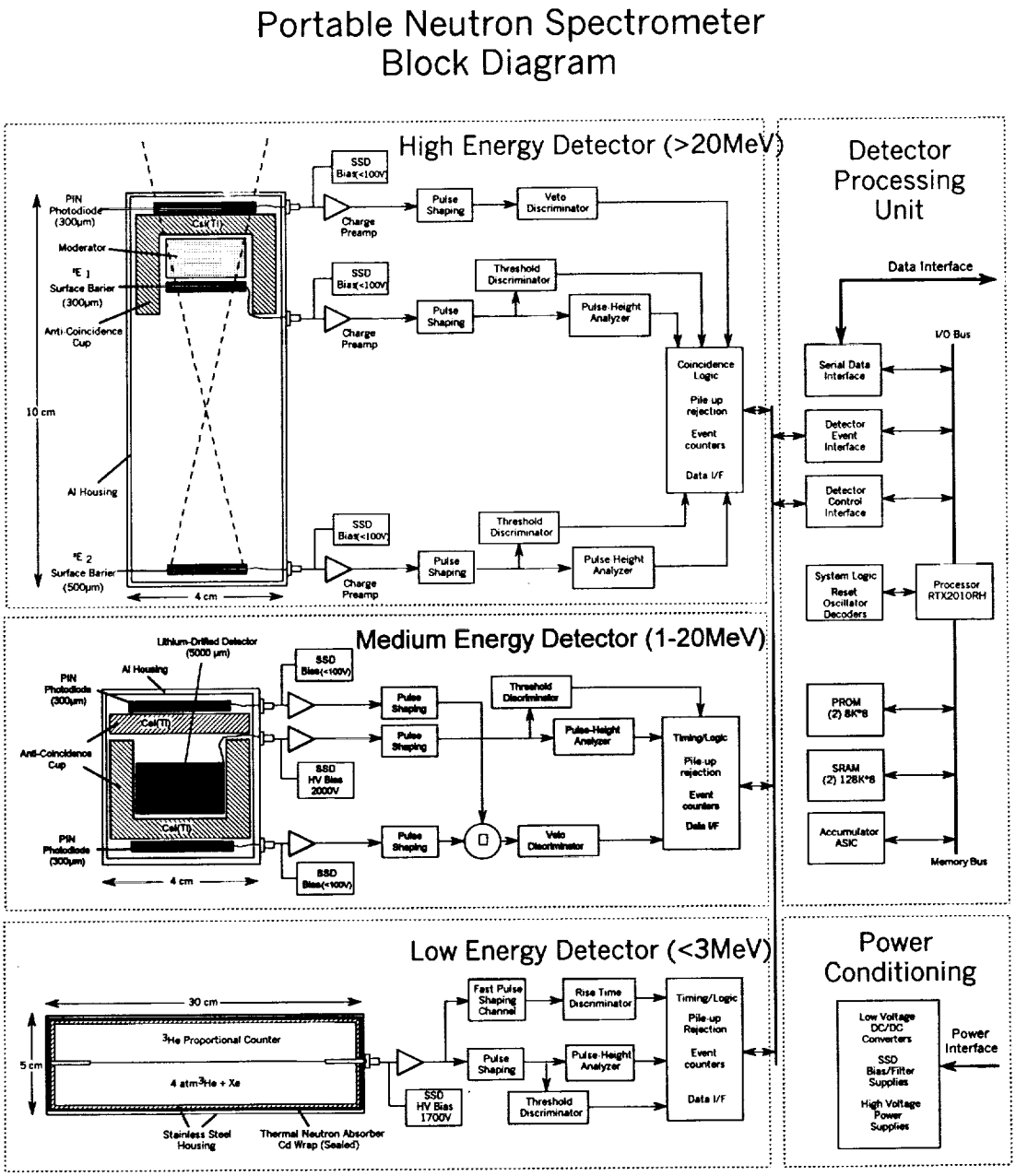


Figure A.8. Portable neutron spectrometer block diagram (now obsolete) as of the 5/13/99 Annual Report. A three-detector instrument was the concept at that time.

H. MANES Block Diagram

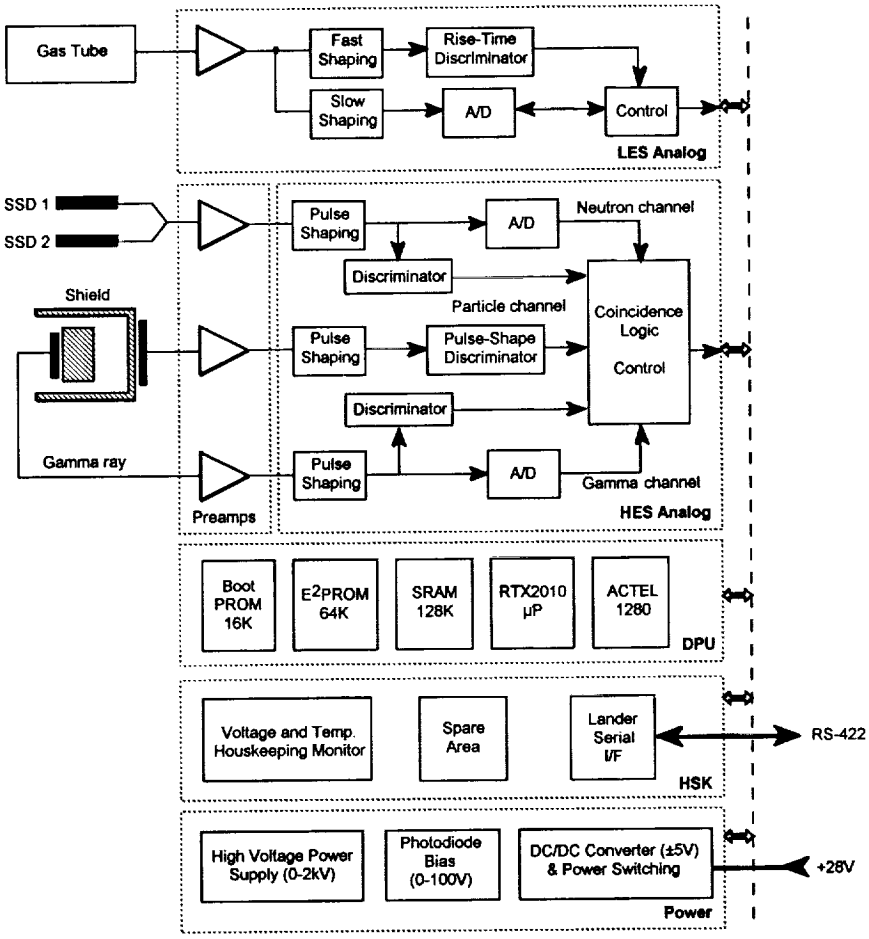


Figure A.9. Detailed block diagram of the MANES instrument showing the use of the dual silicon detectors for high energy neutron measurements. This block diagram shows the simplification in going from a three channel system to a two channel system based on the modeling of neutron-silicon and neutron-polyethylene reactions.

I. Low Energy RARAF Spectrum

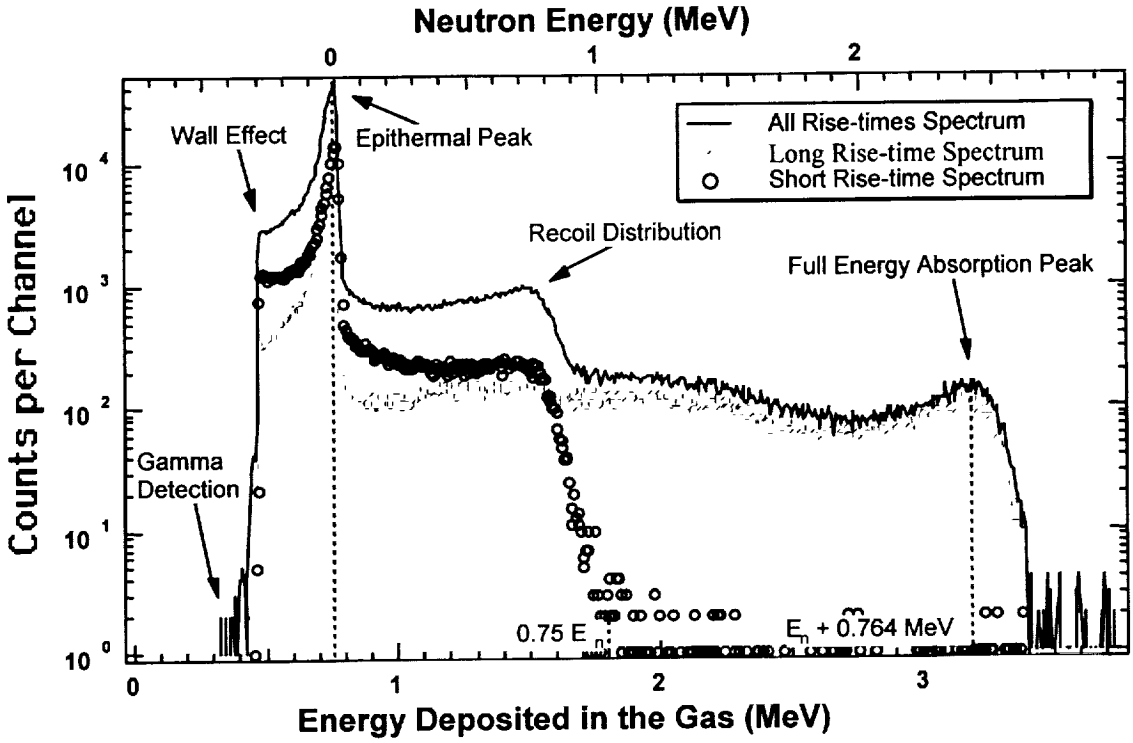


Figure A.10. Monochromatic spectra of the Helium 3 gas tube due to a 2.46 MeV neutron beam at Columbia RARAF in February, June, and October 1999. The full energy capture peak is shown at 2.46 MeV neutron energy (upper abscissa) or 3.22 MeV total energy (lower abscissa).

J. Initial Silicon Detector RARAF Experiments

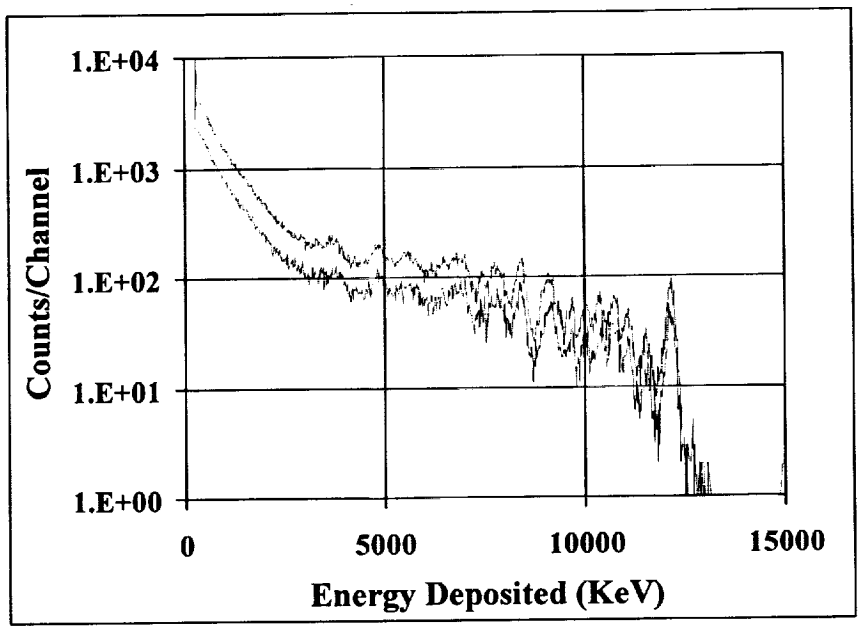


Figure A.11. Energy deposition spectrum due to 14 MeV neutron beam at Columbia RARAF (February 1999) in the 5 mm detector. Each run is not normalized for neutron fluence. The smooth part of the spectrum in Figure A.11. below energies of 2 MeV is the mainly due to neutron-Si elastic reactions; the peaked structures at high energy depositions are due to inelastic channels. These inelastic channels contain protons and alpha particles emitted from excited compound nucleus states formed by the neutron-silicon nuclear reaction.

K. Telescope Efficiency Model Results

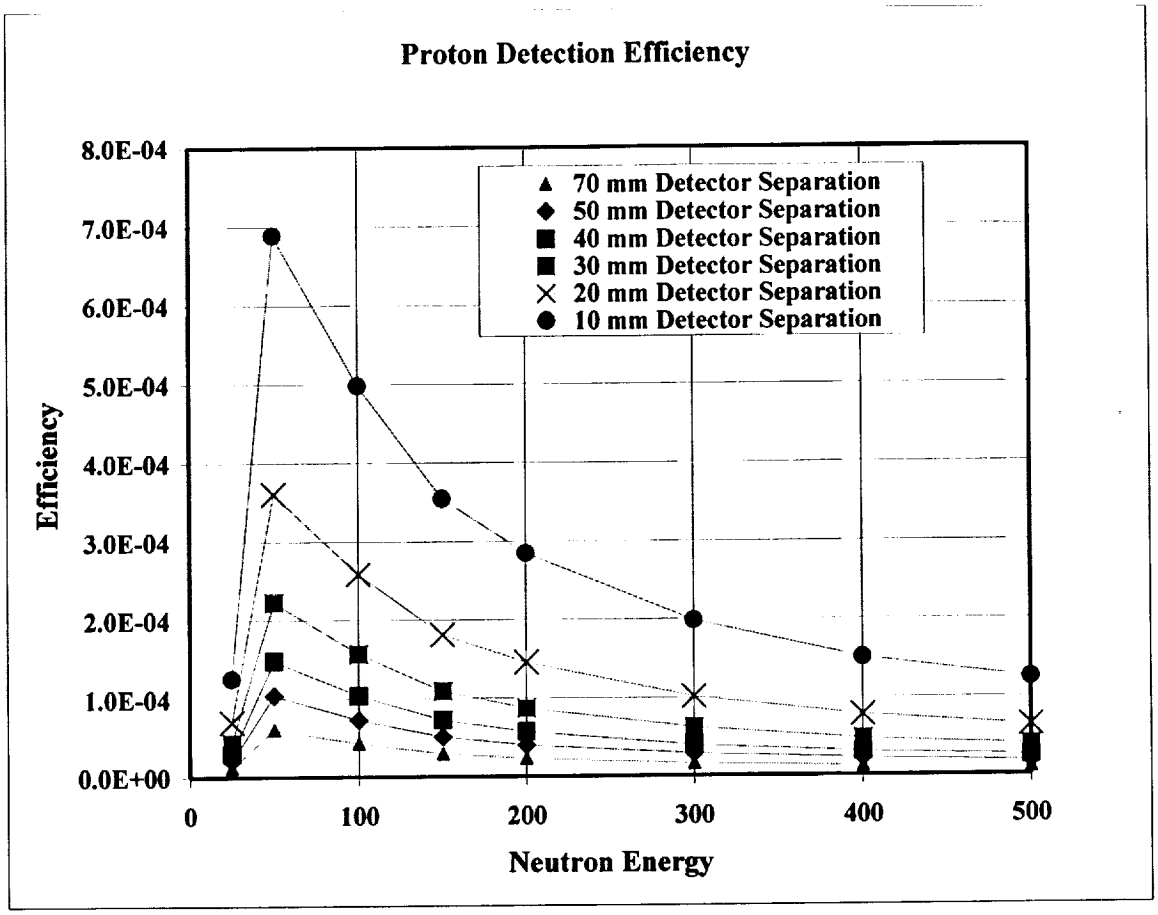


Figure A.12. Model results of high energy proton recoil telescope detection efficiency versus incident neutron energy for various detector separations. The low efficiencies calculated are a combination of the limitations of both the polyethylene moderator/filter (Figure A.7.) and the telescope geometry.

L. Prototype Instrument

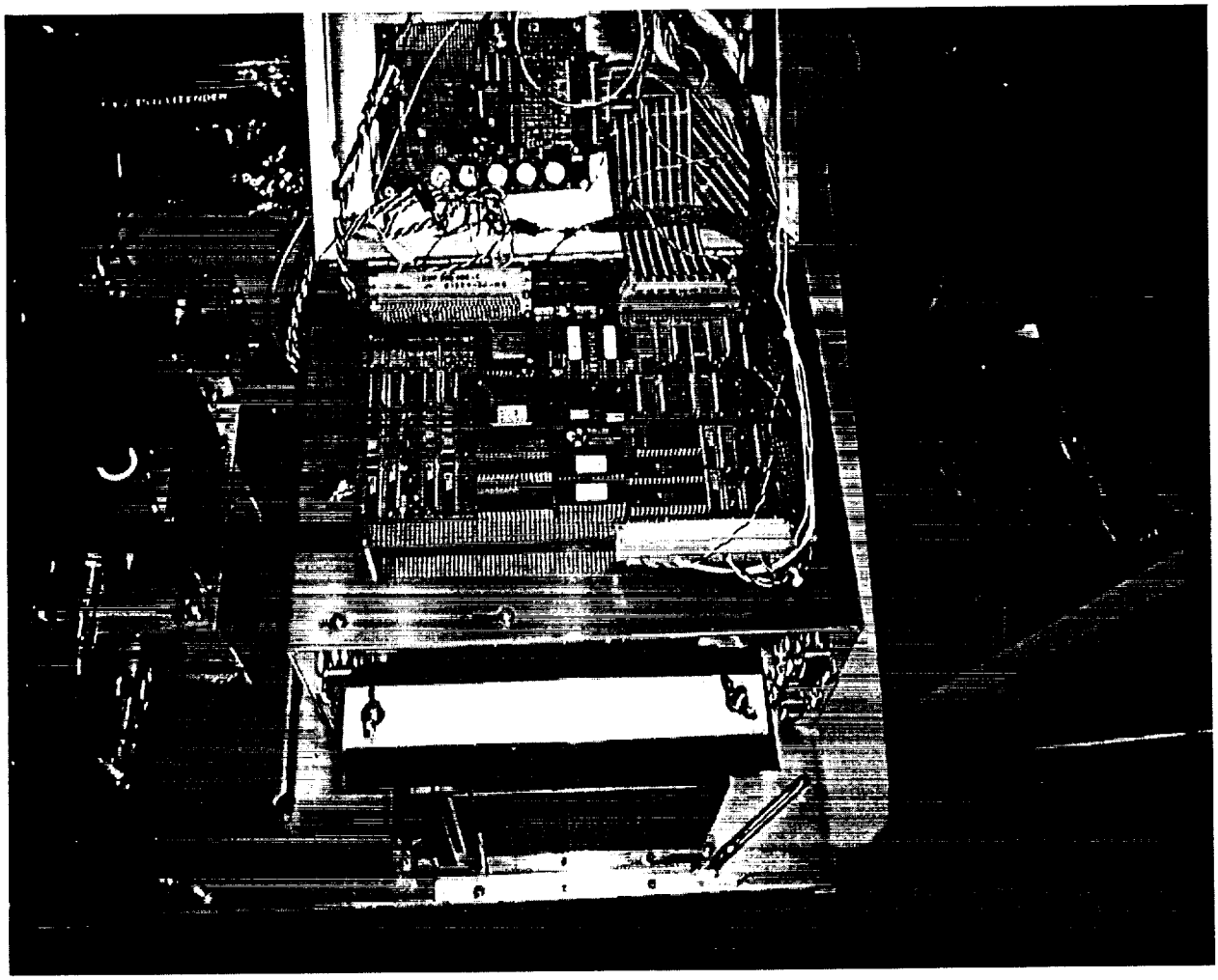


Figure A.13. Top view of prototype instrument developed for high altitude aircraft flights. The ^{3}He gas tube is at the lower left. The battery is the black rectangular object in the lower center below the electronics box. The high energy detector is inside the electronics enclosure in the center of the picture.

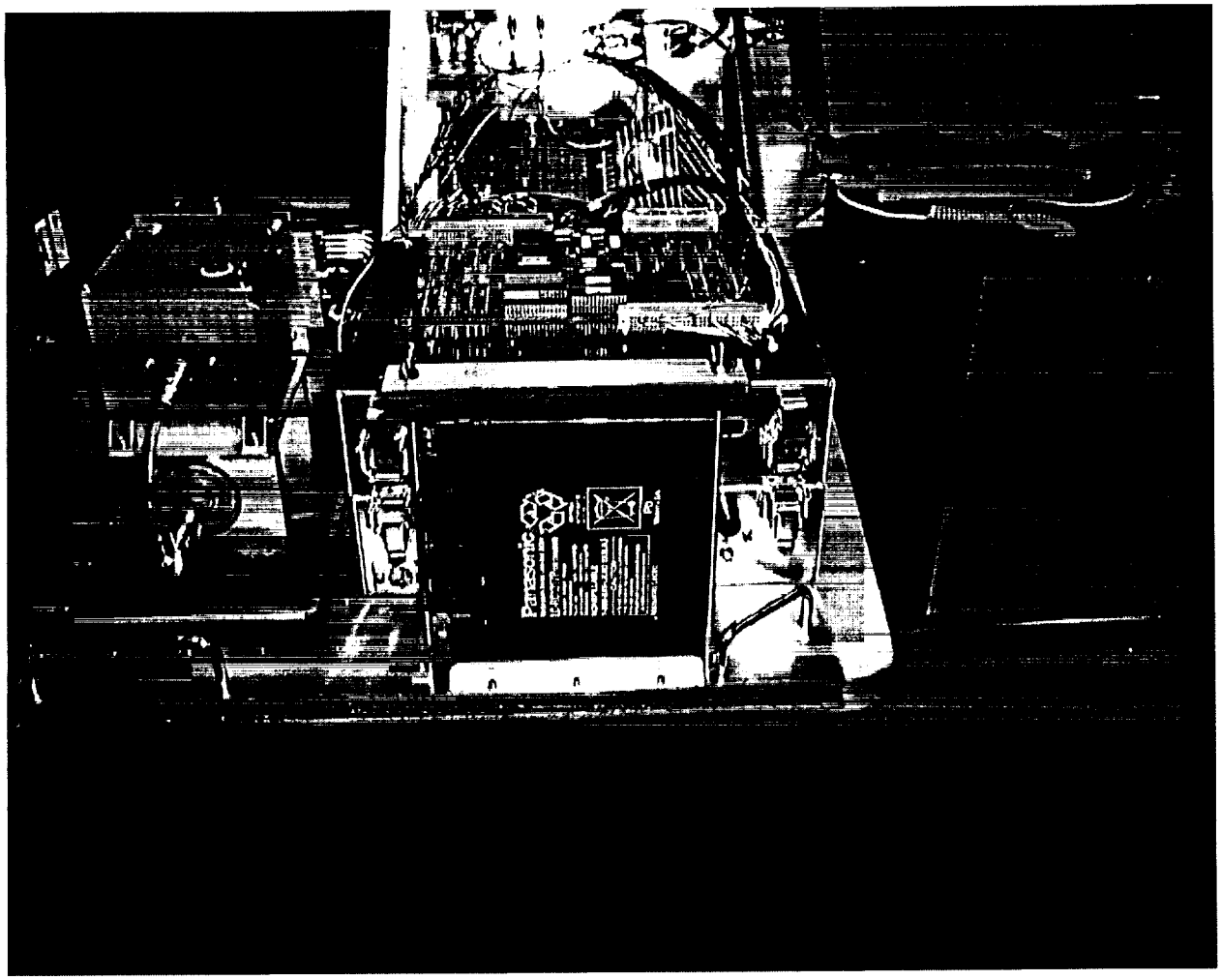


Figure A.14. Front view of prototype instrument developed for high altitude aircraft flights. The battery is clearly seen on the right side of the instrument, with the low energy gas tube and high voltage supply shown on the left.



Figure A.15. Co-investigators David Roth and John Goldsten mounting the prototype hardware into an F-15 aircraft pod, April 2000.

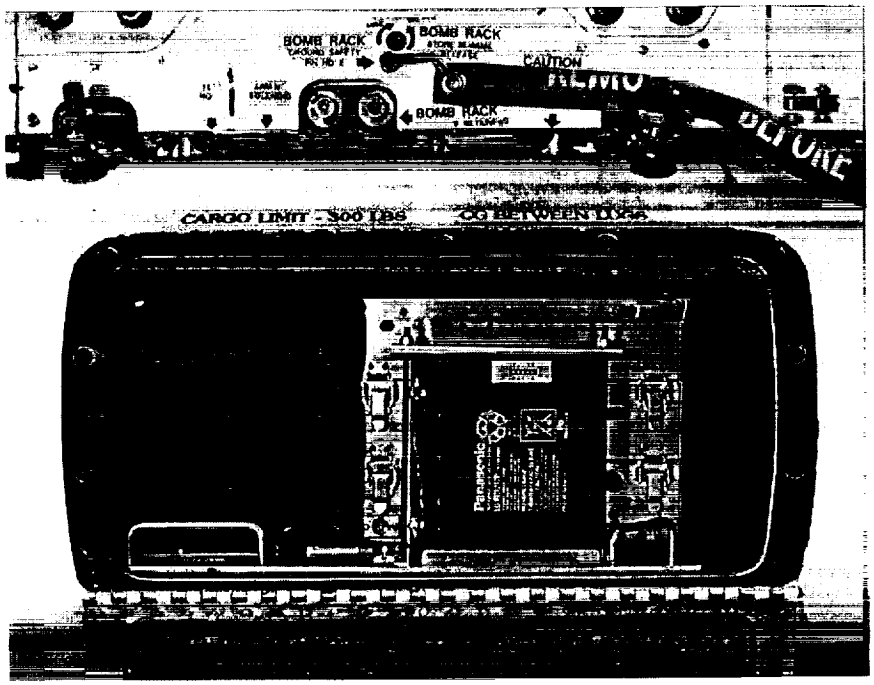
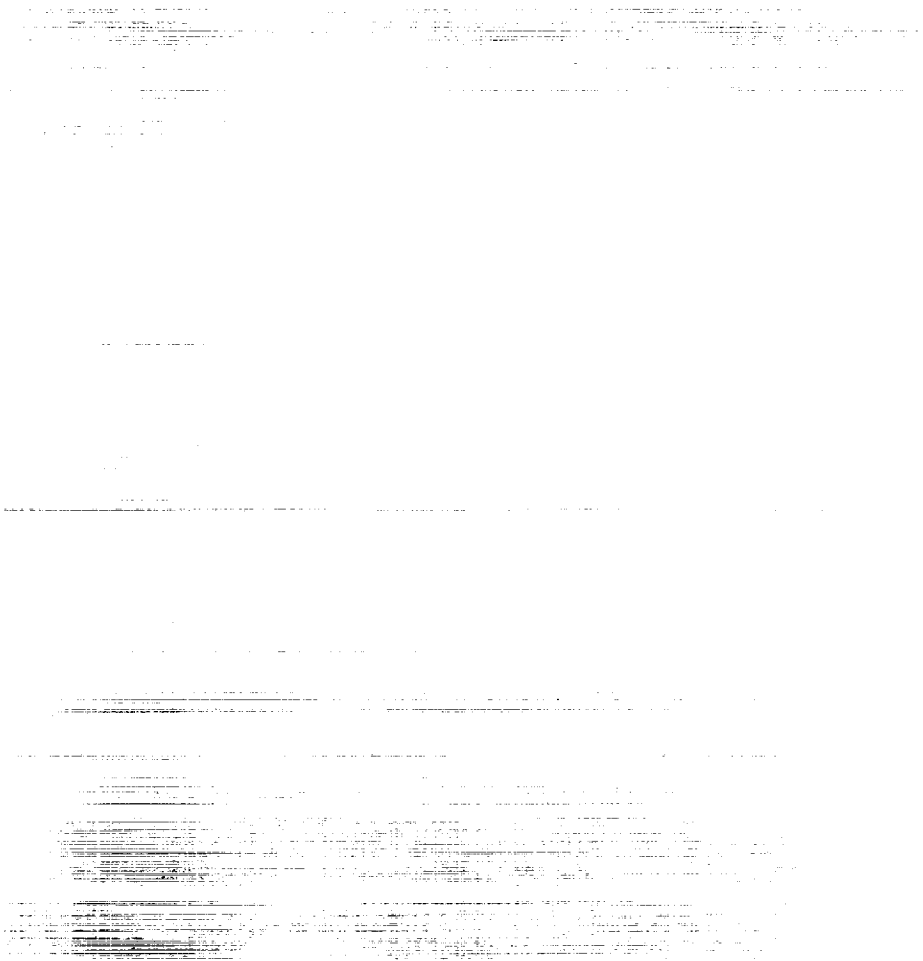
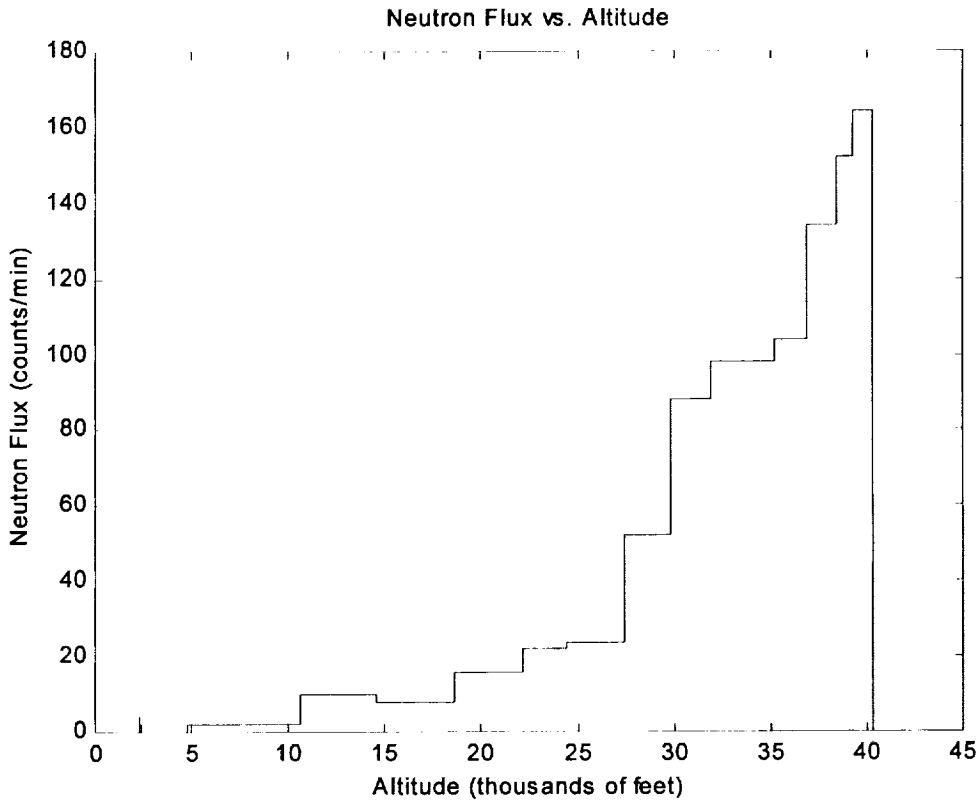


Figure A.16. Close-up of the prototype instrument mounted in the F-15 pod.



M. High Altitude Flight Data



A.17. Count rate in the low energy channel as a function of aircraft altitude for the first prototype instrument flight. At 39,000 feet, the high voltage circuit failed due to corona discharge in spite of successfully completing previous qualification tests to a simulated altitude of 45,000 feet. The prototype instrument is being repaired, and future aircraft flights have been proposed under a follow-on NSBRI grant.

N. Detailed High Energy Spectrometer Design

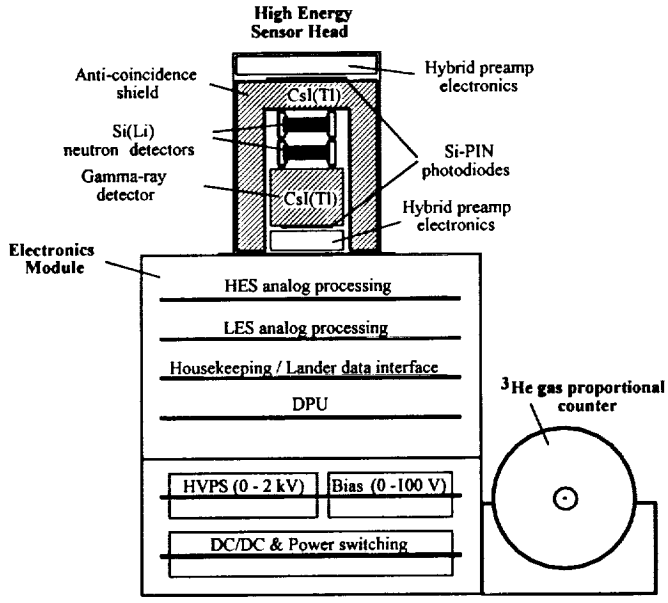


Figure A.18. Proposed design of the sensor head for the MANES High Energy Spectrometer (HES). The diagram also shows the placement of electronics and the Low Energy Spectrometer tube in relation to the HES. This figure illustrates the miniaturization needed for a Mars Lander mission. The HES uses two 5 mm silicon detectors as currently proposed, but may use 7 mm detectors if they become available.

O. Silicon Detector Energy Deposition and Efficiency

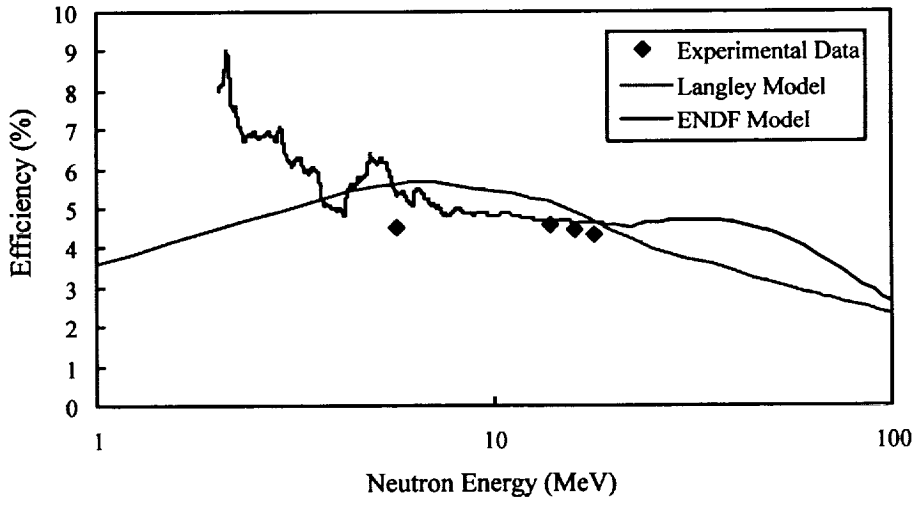


Figure A.19. 5 mm Si(Li) detector efficiency as a function of energy from NASA- and DOE-based cross-section models compared to RARAF experiments. This agreement indicates that MANES can efficiently measure neutron fluence to 50 - 100 MeV. This data confirms that the bulk silicon detector is more efficient than a proton recoil telescope over the energy range of interest.

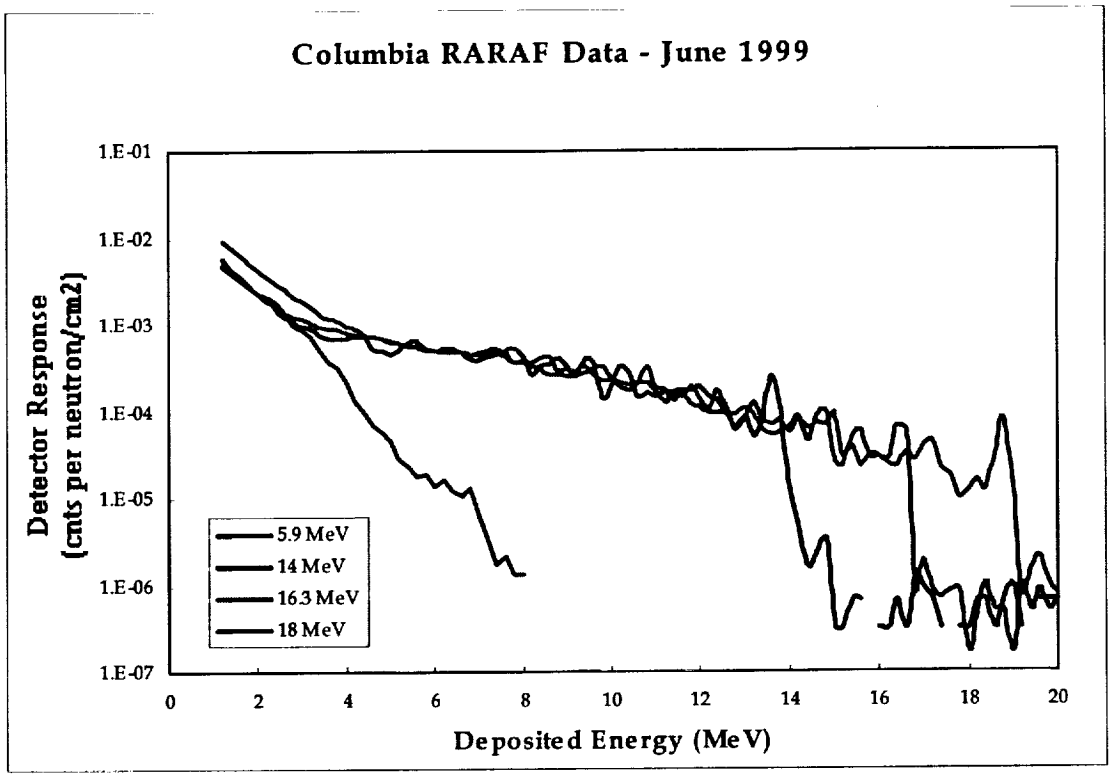


Figure A.20. Energy deposition spectra measured with the 5 mm silicon detector during mono-energetic neutron exposures at Columbia University's RARAF Van de Graaff accelerator. This data shows that energy depositions occur up to the incident neutron energy. These experiments are the basis for model verification and for the development of the instrument response function. The measured efficiencies shown in the previous figure were derived from this data.

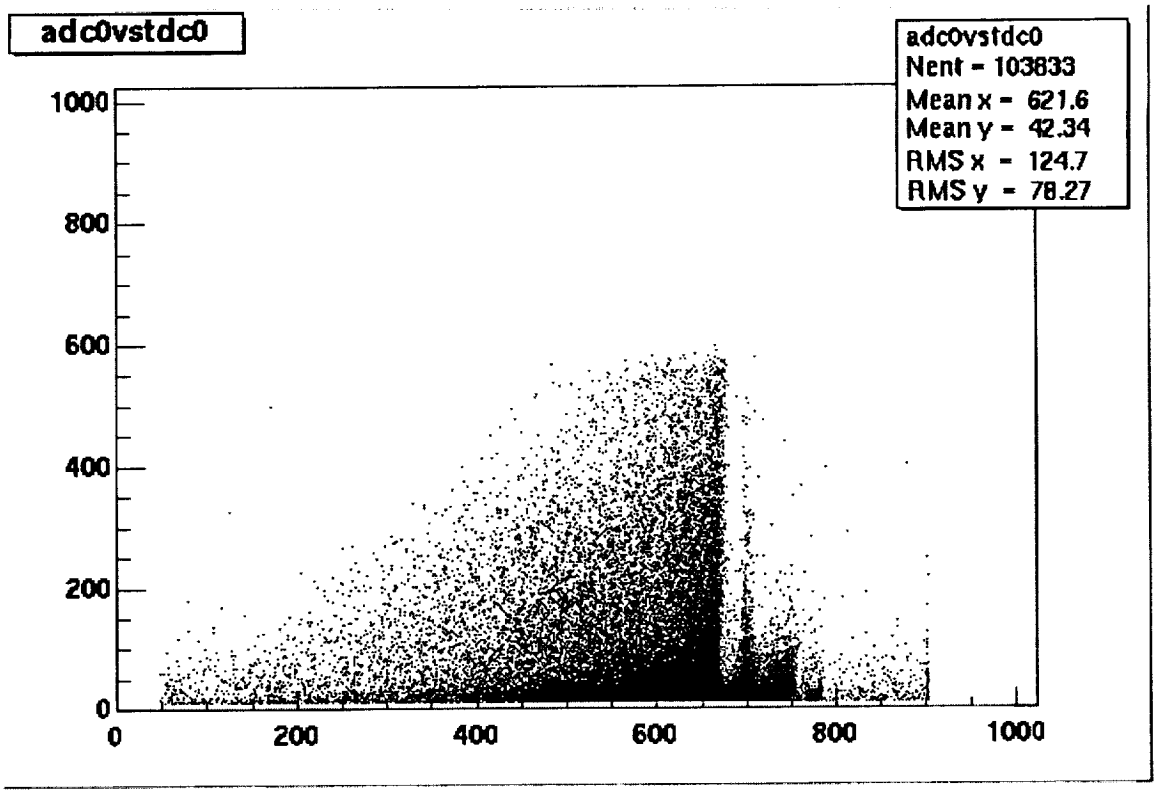


Figure A.21. Raw data from Los Alamos Neutron Science Center experiments, August 2000. The abscissa is time of flight for the incident neutron, from which the incident neutron energy can be calculated. The ordinate is the pulse height detected in the silicon detector for that incident neutron from which deposited energy can be calculated. Black dots indicate the presence of a neutron-silicon interaction for that incident neutron which deposited the given amount of energy. Further analysis of this data is underway, and will lead to the development of the instrument response function.

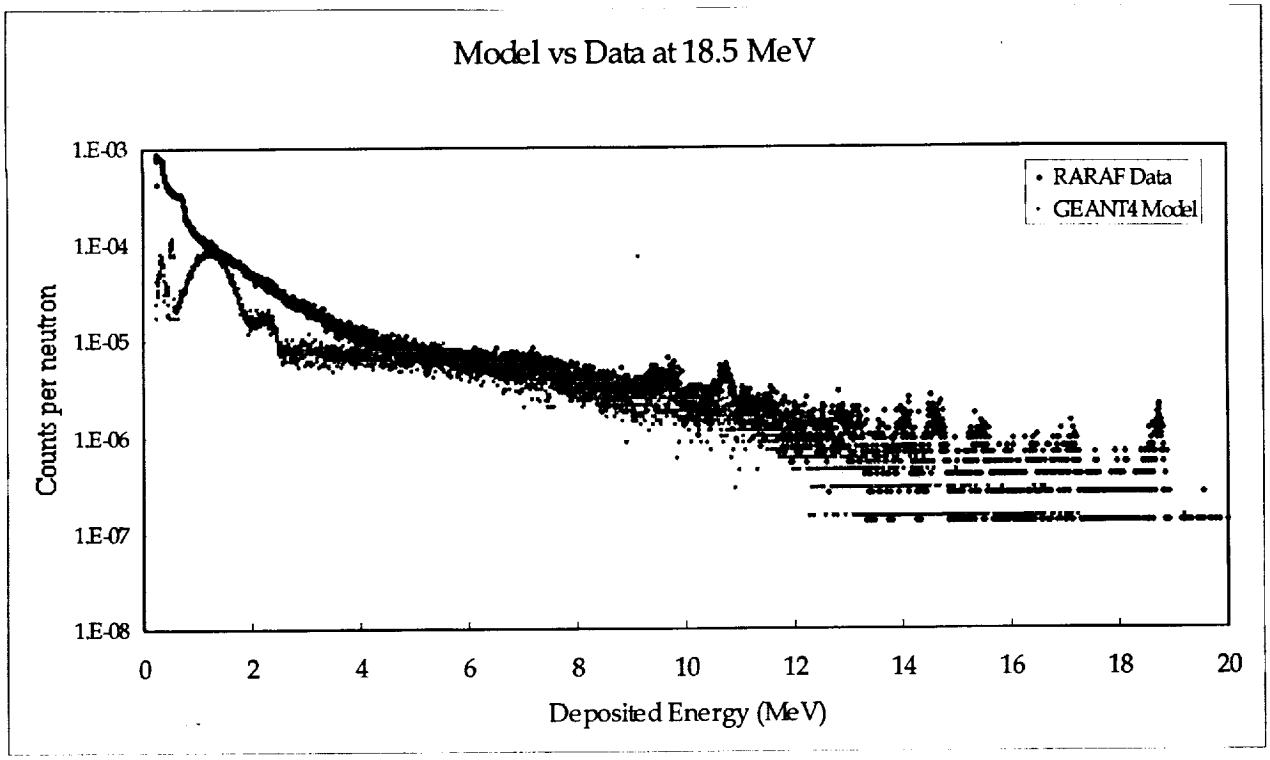


Figure A.22. Comparison of GEANT4 calculated energy deposition spectrum for 18.5 MeV neutrons and the 18.5 MeV data taken at Columbia University RARAF, June 1999. The GEANT4 model accurately predicts the energy deposition spectrum except at the highest energies where the base cross-sections are incomplete and at the lowest energies where the RARAF data may be contaminated with gamma ray background.

Appendix B. Publications/Presentations List

R.H. Maurer, D. R. Roth, R. Fainchtein, J. O. Goldsten, J.D. Kinnison, and A. K. Thompson, "Portable Real Time Neutron Spectrometry," Proceedings of the 1998 Workshop on Predictions and Measurements of Secondary Neutrons in Space, Houston, Texas, 28-30 September 1998.

D. R. Roth, R. H. Maurer, J. D. Kinnison, P. J. McNulty, and M. W. Savage, "Charged Particle and Neutron Solid State Detectors - A Comparison," presented at the 1998 IEEE Nuclear Science Symposium, Toronto, Canada, 9-11 November, 1998.

R.H. Maurer, D. R. Roth, R. Fainchtein, J. O. Goldsten, J. D. Kinnison, and A. K. Thompson, "Portable Real Time Neutron Spectrometry," Proceedings of the First Biennial Space Biomedical Investigators' Workshop, Houston, Texas, 11-13 January 1999, pp500-502.

R.H. Maurer, D. R. Roth, R. Fainchtein, J. O. Goldsten, J. D. Kinnison, and A. K. Thompson, "Portable Real Time Neutron Spectrometry," Proceedings of the Space Technology and Applications International Forum 99, Conference on International Space Station Utilization, Albuquerque, 31 Jan-4 Feb, 1999, American Physical Society CD-ROM.

R.H. Maurer, "Portable Neutron Spectrometer," NSBRI Baltimore Area Luncheon, APL, Laurel, MD, 14 September 1999.

R.H. Maurer and J. O. Goldsten, "MANES (MArtian Neutron Energy Spectrometer) on the Mars 2003 Lander," Mars 2003 Lander Payload Accommodation Meeting, Pasadena, CA, 8 December 1999.

R.H. Maurer, D. R. Roth, R. Fainchtein, J. O. Goldsten and J. D. Kinnison, "Portable Real-Time Spectrometry II," NSBRI Biannual Retreat, Houston, Texas, 11 January 2000.

R.H. Maurer, D. R. Roth, R. Fainchtein, J. O. Goldsten and J. D. Kinnison, "Portable Real-Time Spectrometry II," Proceedings of the Space Technology and Applications International Forum 00, Conference on the International Space Station Utilization, Albuquerque, NM, 31 Jan-3Feb 2000, American Physical Society CD-ROM.

R.H. Maurer, R. Fainchtein, R. E. Gold, J. O. Goldsten, J. D. Kinnison, D. R. Roth, G. Badhwar and J. F. Dicello, "MANES (Martian Neutron Energy Spectrometer) Definition Phase Status Report," First Meeting of the Mars 2003 Program Science Group, Pasadena, CA, 24-25 February 2000.

R.H. Maurer, R. Fainchtein, R. Gold, J.O. Goldsten, J. D. Kinnison, D.R. Roth, G. Badhwar and J.F. Dicello, "MANES (MArtian Neutron Energy Spectrometer) Program Status Report," NASA Headquarters Review, Washington, D.C. , 29 March 2000.

R.H. Maurer, R. Fainchtein, J. Goldsten, J. Kinnison, D. Roth, "MArtian Neutron Energy Spectrometer: An Instrument for the Mars 2003/5 Lander," Fourth IAA International Conference on Low Cost Planetary Missions, APL, Laurel, MD, 5 May 2000.

R.H. Maurer, J.D. Kinnison, D.R. Roth, J. Miller, L. Heilbronn, C. Zeitlin, R. Singleterry, "Development of a Neutron Spectrometer to Assess Biological Radiation Damage Behind Spacecraft Materials," NASA Microgravity Materials Science Conference, Huntsville, AL, 7 June 2000.

R.H. Maurer, D.R. Roth, J.D. Kinnison, J.O. Goldsten, R. Fainchtein, G. Badhwar, "MArtian Neutron Energy Spectrometer (MANES)," Concepts and Approaches for the Robotic Exploration of Mars, Lunar and Planetary Institute, Houston, TX, 18 July 2000.

NSBRI RESEARCH PROGRAM
SYNERGY PROJECTS

Cohen, R. J.	**PI**	**MIT**	**Alterations in Cardiovascular Regulation and Function During Long-Term Simulated Microgravity (Cardiovascular Alterations – Bone Loss)**
LeBlanc, A.	CO-I	Baylor	
Ramsdell, C. D.	CO-I	Harvard	
Shackelford, L.	CO-I	NASA JSC	
Yelle, J. M.	CO-I	NASA JSC	
Dijk, D.	**PI**	**Harvard**	**Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and the Renin-Angiotensin System (Cardiovascular Alterations – Human Performance)**
Cajochen, C.	CO-I	Harvard	
Williams, G. H.	CO-I	Harvard	
Ramsdell, C. D.	**PI**	**Harvard**	**Visual- and Vestibular-Autonomic Influence on Short-Term Cardiovascular Regulatory Mechanisms (Cardiovascular Alterations – Neurovestibular Adaptation)**
Beall, A. C.	CO-I	MIT	
Mullen, T. J.	CO-I	Harvard	
Oman, C. M.	CO-I	MIT	
Paloski, W.	CO-I	NASA JSC	
Wood, S.	CO-I	Baylor	
Yelle, J. M.	CO-I	NASA JSC	
Mullington, J. M.	**PI**	**Harvard**	**Sustained Partial Sleep Deprivation: Effects on Immune Modulation & Growth Factors (Human Performance – Immunology, Infection & Hematology – Muscle Alterations)**
Butel, J. S.	CO-I	Baylor	
Dinges, D. F.	CO-I	Penn	
Lednicky, J. A.	CO-I	Baylor	
Ling, P. D.	CO-I	Baylor	
Schwartz, R. J.	CO-I	Baylor	

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Annual Project Report

1. **Project Title:** Alterations in Cardiovascular Regulation and Function During Long-Term Simulated Microgravity

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Baylor College of Medicine

C. Janice M. Yelle
Johnson Space Center

D. Linda Shackelford, Ph.D.
Johnson Space Center

Richard J. Cohen, M.D., Ph.D.
Whitaker Professor of Biomedical Engineering

Date

EXECUTIVE SUMMARY

The Cardiovascular Alterations Team is conducting studies of hemodynamic regulation and susceptibility to arrhythmias resulting from sixteen days of simulated microgravity exposure. In these studies very intensive measurements are made during a short duration of bed rest. In this collaborative effort are making many of the same measurements, however much less frequently, on subjects who are exposed to a much longer duration of simulated microgravity.

Alterations in cardiovascular regulation and function that occur during and after space flight have been reported. These alterations are manifested, for example, by reduced orthostatic tolerance upon reentry to the earth's gravity from space. However, the precise physiologic mechanisms responsible for these alterations remain to be fully elucidated. Perhaps, as a result, effective countermeasures have yet to be developed. In addition, numerous reports from the past 30 years suggest that the incidence of ventricular arrhythmias among astronauts is increased during space flight [Charles *et al.*, 1994, Fritsch-Yelle, *et al.*, 1998]. However, the effects of space flight and the associated physiologic stresses on cardiac conduction processes are not known, and an increase in cardiac susceptibility to arrhythmias has never been quantified.

In this project we are applying the most powerful technologies available to determine, in a ground-based study of long duration space flight, the mechanisms by which space flight affects cardiovascular function, and then on the basis of an understanding of these mechanisms to develop rational and specific countermeasures. To this end we are conducting a collaborative project with the Bone Demineralization/Calcium Metabolism Team of the National Space Biomedical Research Institute (NSBRI). The Bone Team is conducting bed rest studies in human subjects lasting 17 weeks, which provides a unique opportunity to study the effects of long duration microgravity exposure on the human cardiovascular system. We are applying a number of powerful new methods to these long term bed rest subjects, including cardiovascular system identification (CSI), microvolt level T wave alternans analysis, and cardiac magnetic resonance imaging to assess non-invasively the effects of simulated long duration space flight on the cardiovascular system.

CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate, arterial blood pressure (ABP), and instantaneous lung volume (ILV – respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of all the physiologic mechanisms coupling these signals, CSI provides a model of the closed-loop cardiovascular regulatory state in an individual subject. The model includes quantitative descriptions of the heart rate baroreflex as well as other important physiologic mechanisms. With an additional non-invasive measurement of stroke volume (SV – ultrasound Doppler method), the model may be extended to also include the characterization of the peripheral resistance baroreflex – which may play a central role in the development of orthostatic intolerance – and measures of systolic and diastolic function.

To determine whether simulated long-term space flight increases the risk of developing life-threatening heart rhythm disturbances such as sustained ventricular tachycardia (defined as ventricular tachycardia lasting at least 30 seconds or resulting in hemodynamic collapse) and ventricular fibrillation, we are applying a powerful new non-invasive technology, developed in Professor Cohen's laboratory at MIT, for the quantitative assessment of the risk of life-threatening ventricular arrhythmias. This technology involves the measurement of microvolt levels of T wave alternans during exercise stress. In addition, we are obtaining 24-hour Holter

monitoring to detect non-sustained ventricular tachycardia and to assess heart rate variability. Finally, in order to investigate the effect of long duration microgravity on cardiac mass, cardiac magnetic resonance images are being obtained before and after the bed rest period.

To date, measurements for CSI, 24-hour Holter monitoring, and cardiac magnetic resonance imaging have been made on seven long-term bed rest subjects. Measurements for TWA analysis have been made in four of these subjects. The studies are still ongoing, and only preliminary analysis of the data has been completed. During Year 3 we will complete the analysis of the data generated in this study.

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I. RESEARCH PLAN SUMMARY

A. HYPOTHESES, OBJECTIVES & SPECIFIC AIMS FROM ORIGINAL PROPOSAL

Hypotheses:

- The peripheral resistance baroreflex, heart rate baroreflex, and other autonomically mediated physiologic mechanisms are altered as a result of exposure to simulated microgravity. Alterations in these physiologic mechanisms play a central role in orthostatic intolerance.

- Cardiac systolic and diastolic function are altered as a result of exposure to simulated microgravity and also contribute to reduced orthostatic tolerance.

- Countermeasures, which are developed based on the above alterations (*e.g.*, α-sympathetic agonists for reduced peripheral resistance baroreflex responsiveness, digitalis for decreased systolic function), will ameliorate the cardiovascular deconditioning process and in particular, orthostatic intolerance.

- There is a decrease in cardiac mass as a result of exposure to long term simulated microgravity.

- There is an alteration in cardiac electrical function, as a result of exposure to long duration simulated microgravity, resulting in increased susceptibility to ventricular arrhythmias.

- The combination of advanced non-invasive technologies employed in this study will detect these subtle alterations.

- Countermeasures, which are developed based on the above alterations (e.g., specific anti-arrhythmic agents or electrolyte therapy) will ameliorate the increased susceptibility to ventricular arrhythmias as a result of space flight.

Specific Aims:

There are two general aims of this project: 1) to apply CSI to investigate quantitatively and non-invasively alterations in cardiovascular regulation and function during and after long term simulated microgravity; 2) to apply microvolt level T wave alternans analysis, along with other non-invasive methods based on SAECG, QT Dispersion, and 24 hour Holter monitoring, to investigate the impact of prolonged exposure to simulated microgravity on susceptibility to ventricular arrythmias. The specific aims of this project are as follows:

- To establish which specific physiologic mechanisms are responsible for the alterations in integrated hemodynamic behavior due to simulated microgravity.
- To determine the nature, time course, and sequence of changes in cardiovascular regulation and function during and after simulated microgravity.
- To determine what countermeasures may be effective in ameliorating the alterations associated with microgravity, especially reduced orthostatic tolerance.
- To determine if susceptibility to ventricular arrhythmias is increased with exposure to long duration simulated microgravity.
- To determine to what extent prolonged exposure to simulated microgravity diminishes cardiac mass.
- To hypothesize potential countermeasures provided that susceptibility to ventricular arrhythmias is increased after simulated microgravity.

B. MODIFICATIONS REQUIRED UPON SELECTION

None.

C. SUMMARY OF PROGRESS IN YEAR 1.

The Bone Demineralization/Calcium Metabolism Team's long duration bed rest study is underway. Subject recruitment for this study is ongoing, and a number of subjects were identified for the summer start dates.

This Synergy project was selected for funding in April of 1998, however these funds were not released until June of 1998. Therefore implementation of the protocol was slightly delayed. Existing equipment and personnel for the acquisition of beat to beat stroke volume, heart rate, blood pressure and instantaneous lung volume, as required for CSI analysis, was identified and positioned to begin experiments. In addition, the protocols and personnel for the acquisition and analysis of cardiac magnetic resonance images were identified. The CH-2000 (Cambridge Heart, Inc.), required for T-wave alternans was

purchased and delivered to the Johnson Space Center Cardiovascular Laboratory, and a supine bicycle modification required for this project was implemented.

Funding for this project was received towards the end of year 1 and shortly thereafter the first data collection sessions on the long-term bed rest subjects were begun.

D. ACTIONS TAKEN IN RESPONSE TO LAST YEAR'S CRITIQUE

None.

I. DETAILED RESEARCH PLAN – YEAR 2

We are acquiring the data necessary for CSI, T wave alternans, and 24-hour Holter monitoring analyses during the experimental protocol of the Bone Demineralization/Calcium Metabolism Team. The protocol is divided into five 22-week testing periods and involves a total of 20 normal test subjects (5 males and 5 females in the resistive exercise group and 5 males and 5 females in the control group). Each 22 week period is divided into four phases: phase I (week 1) for orientation and metabolic equilibration on a controlled diet; phase II (weeks 2-3) for metabolic balance testing, familiarization with the strength training protocol, and baseline measurements, and baseline testing for all measurements; phase III (weeks 4-20) for the bed rest portion of the study; and phase IV (weeks 21-22) for the post-bed rest recovery portion. During the one-year period that this study will be conducted, approximately 10 subjects will complete the protocol, and will be studied by the Cardiovascular Alterations Team.

In particular, we are acquiring data for CSI and T wave alternans once during a fixed time in phase II, once every four weeks at a fixed time during phase III, and once at a fixed time during phase IV. 24 hour Holter monitor data is gathered twice during the study, once during phase II, and once during phase IV.

Data acquisition for CSI involves non-invasively measuring and recording surface ECG, ABP, ILV, and SV signals for each subject using an on-line dedicated data sampling and analysis program. The ABP signal is measured from the middle finger of the left or right hand using a Finapres blood pressure monitor (Ohmeda, Inc.). The ILV signal is measured with a Respitrace system two belt chest-abdomen inductance plethysmograph (Ambulatory Monitoring Systems, Inc.) and calibrated with an 800 cc inflatable spirobag. The SV signal is measured by an ultrasound Doppler method [Eriksen & Walløe, 1990]. A bi-directional ultrasound Doppler velocimeter is operated in pulsed mode at 2 MHz with a handheld transducer. The ultrasound beam will be directed from the suprasternal notch toward the aortic root, and the sample volume will be positioned centrally in the aorta approximately one to two cm above the aortic valve. The constant diameter of the rigid aortic ring is determined by parasternal sector-scanner imaging. SV is calculated as the product of the value obtained by the numerical integration of the continuously recorded instantaneous maximal blood flow velocity during each RR interval and the area of the aortic orifice. For each CSI data acquisition period, we record these signals for 8 minutes as subjects breathe according to a random interval breathing protocol.

The data is transferred to MIT for subsequent data processing and CSI analysis. The processed data is also used to calculate the power spectra of the four measured variables. Particular attention is paid to relative changes in low and high frequency bands and their interpretation in terms of autonomic activity. By comparing the changes in variability in all of these signals

throughout the protocols, we obtain a further understanding of the mechanisms underlying cardiovascular deconditioning.

We are measuring the electrocardiographic data necessary for microvolt level T wave alternans during horizontal bicycle exercise. The subject controls his pedaling rate at 1/3 of his heart rate during the bicycle stress for noise reduction purposes. The exercise stress test is conducted at up to 70% of the subject's predicted maximum heart rate (maximum predicted heart rate is defined as 220 bpm minus the patient's age in years). The data for 24 hour Holter monitoring is collected during a 24 hour period following the above data collection periods for CSI and T wave alternans.

For microvolt level T wave alternans a Frank orthogonal electrocardiographic lead system is used. All ECG measurements will be performed with the CH 2000 (Cambridge Heart) system. The CH 2000 is capable of performing on-line analysis for microvolt level T wave alternans. We determine whether sustained alternans (continuous alternans with $K > 3$ and $V_{alt} > 1.9$ microvolts) occurs above a subject specific heart rate threshold [Rosenbaum *et al.*, 1996]. When sustained alternans is present we determine the heart rate threshold. In this way we determine whether exposure to simulated space flight causes sustained alternans to occur, and if so, at what characteristic heart rate threshold.

Cardiac magnetic resonance images (MRI) are being performed on each subject studied during phase II and phase IV. These images are acquired by the Bone Demineralization / Calcium Metabolism Team under the guidance G. Wesley Vick, III, M.D., Ph.D. of the Section of Pediatric Cardiology at Baylor College of Medicine who has extensive experience with cardiac MRI acquisition and analysis.

II. PROGRESS, RESULTS & ACCOMPLISHMENTS – YEAR 2

Below is a breakdown for each subject and tests performed to date. Some studies are still in progress. We anticipate collecting data on at least three more subjects during Year 2 and the beginning of Year 3.

MH - female control (study dates: September 1998 - January 1999)

CSI - Pre-bed rest (supine only), 5x during bed rest, 2x post bed rest (supine & standing).
TWA – none (equipment not available).
Holter - pre and post bed rest.

DFH - male resistive exercise (study dates: September 1998 - January 1999)

CSI - Pre-bed rest (supine only), 4x during bed rest, 2x post bed rest (supine & standing).
TWA – none (equipment not available).
Holter - pre and post bed rest.

SS - female control (study dates: September 1998 - January 1999)

CSI - Pre-bed rest (supine only), 4x during bed rest, 2x post bed rest (supine & standing).
TWA – none (equipment not available).
Holter - pre and post bed rest.

VS - male resistive exercise (study dates: November 1998 - April 1999)

CSI - Pre-bed rest (supine only), 4x during bed rest, 2x post bed rest (supine & standing).
TWA - 2x during bed rest (weeks 13& 17), 2x post bed rest
Holter - pre and post bed rest

MC - female control (study dates: January 1999 - May 1999)

CSI - Pre-bed rest (supine & standing), 3x during bed rest (weeks 5, 9 & 17).
TWA - 4x during bed rest (weeks 5 (too noisy), 6 (per Dr. Cohen's request, 9 & 17).
Holter - pre bed rest.

MSC - male control (study dates: February 1999 - June 1999)

CSI - Pre-bed rest (supine & standing), 2x during bed rest (weeks 5 & 9).
TWA - pre-bed rest, 2x during bed rest (weeks 5 & 9).
Holter - pre bed rest.

SNH - female resistive exercise (study dates: April 1999 – August 1999)

CSI - Pre-bed rest (supine & standing).
TWA - pre-bed rest.
Holter - pre bed rest.

To date, only preliminary analysis of the TWA data gathered to date from this study has been completed. These results are presented in the annual project report for the *Non-Invasive Assessment of Susceptibility to Ventricular Arrhythmias during Simulated Microgravity* project. Some of the completed CSI data sets from the long-term bed rest study have been transferred to MIT and are currently being digitized and processed. CSI analysis is not yet complete for these data, however some CSI analysis has been completed from the short-bed rest studies being conducted in Boston. These results are presented in the annual project report for the *Alterations in Cardiovascular Regulation and Function during Simulated Microgravity* project.

III. IMPLICATIONS FOR FUTURE RESEARCH & PLAN FOR YEAR 3

We anticipate completing the acquisition and analysis of data for this study during Year 3. The overarching aim of this research is to gain a better understanding of pathophysiologic cardiovascular changes resulting from long-term microgravity exposure so that effective countermeasures may be developed. While current research is mandated to be limited to ground-based studies, the near term focus is clearly on space-based studies. To this end we are using powerful minimally invasive and non-invasive techniques in these ground-based studies that can readily be adapted for in-flight use aboard the Space Shuttle or Space Station.

Cardiovascular system identification is a powerful methodology for assessing non-invasively closed-loop hemodynamic regulation. T-wave alternans analysis is a powerful methodology for assessing non-invasively changes in cardiac electrical stability. In response to the NASA

Research Announcement of 1998, a protocol very similar to the one in this project was submitted for the evaluation of astronauts before and after exposure to microgravity. In addition, most of this protocol can be conducted in actual microgravity conditions, and the methodologies used here can be further used to test the effectiveness of potential countermeasures under actual space flight conditions.

The Bone Demineralization/Calcium Metabolism Team is conducting long duration human horizontal bed rest studies in order to validate their resistive exercise protocol as a countermeasure to microgravity induced bone density losses in a ground based study prior to its implementation in flight. These studies involve a 17-week bed rest period, which is about the same amount of time Mir crews currently spend in microgravity. As mentioned above, during these longer duration space flights, there have been more reports of cardiac arrhythmias than during shorter space flights. As it appears that the frequency of cardiac arrhythmias may increase with increasing duration of microgravity exposure, these long duration bed rest studies are providing an excellent opportunity for the Cardiovascular Alterations Team to study the effects of long duration simulated microgravity exposure on cardiovascular deconditioning and susceptibility to ventricular arrhythmias.

The Bone Demineralization/Calcium Metabolism Team is benefiting from the collaboration in that it gains the opportunity to acquire cardiac magnetic resonance images in addition to the magnetic resonance images of the legs and lumbar spine they originally proposed to acquire. Many of their current acquisition modes and image processing techniques are quite similar to those needed for cardiac magnetic resonance image acquisition, and this is a natural extension of their study. Members of the Bone Demineralization/Calcium Metabolism Team have already been involved in gathering magnetic resonance images of astronauts on landing day using a portable MRI brought to the Kennedy Space Center, and will continue acquiring such images in the future following longer duration missions. Their ability to include cardiac MRI in their repertoire adds considerable value to the post-flight database they are generating.

In future years, the Cardiovascular Alterations Team plans to conduct pre-, intra- and post-flight studies of astronauts exposed to long stays in microgravity including CSI and T wave alternans analysis to quantify any increased susceptibility to ventricular arrhythmias that may result from these longer exposures. Correlating these findings with changes in cardiac mass using cardiac MRI will be invaluable.

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Eriksen M, Walloe L. Improved method for cardiac output determination in man using ultrasound Doppler technique. Medical & Biological Engineering & Computing 1990;28: 555-560.

Fritsch-Yelle JM, Leuenberger UA, D'Aunno DS, Rossum AC, Brown TE, Wood ML, Josephson ME, Goldberger AL. An episode of ventricular tachycardia during long-duration spaceflight.. American Journal of Cardiology, 81(11): 1391-1392, 1998.

APPENDIX A – Publications

APPENDIX B – Other Support

Other Support: Richard J. Cohen, M.D., Ph.D.

ACTIVE

Principal Investigator: Richard J. Cohen

Source: National Space Biomedical Research Institute (Technology Development Team)

Title of Project: Instrumentation for Non-Invasive Assessment of Cardiovascular Regulation

Dates of Approved/Proposed Project: October 1, 1997 - September 30, 2000

Total Costs Current Year: $112,640

Brief Description: Development of non-invasive automated instrumentation to perform cardiovascular system identification by analysis of beat-to-beat variation in cardiovascular signals.

ACTIVE

Principal Investigator: Richard J. Cohen

Source: NASA

Title of Project: NASA Center for Quantitative Cardiovascular Physiology, Modeling and Data Analysis

Dates of Approved/Proposed Project: June 1, 1998 - May 31, 2001

Total Costs Current Year: $200,000

Brief Description: Center grant to develop quantitative techniques to model and analyze alterations in cardiovascular physiology resulting from space flight. Includes research and educational components.

APPENDIX C – Grant Submissions

Grant submissions related to the current project pending funding.

1. NASA: Characterization of Autonomic Function, Sodium Handling, and Nitric Oxide Physiology in Astronauts and Patients Known to be Susceptible to Orthostatic Intolerance. J Yelle (JSC, PI) – Williams (BWH, co-investigator)

2. NASA: Quantitative Analysis of Cardiovascular and Renal-Endocrine Function in Astronauts Before and After Space Flight. RJ Cohen (MIT, PI)







APPENDIX D – Publications Enclosed

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

Synergy Proposal
Annual Report, May 1999

Research Teams: HUMAN PERFORMANCE FACTORS, SLEEP AND CHRONOBIOLOGY TEAM
CARDIOVASCULAR ALTERATIONS TEAM

Study Name: **Acute Total and Chronic Partial Sleep Deprivation: Effects on Neurobehavioral Function, Waking EEG and the Renin-Angiotensin System**

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_____
Date

2. EXECUTIVE SUMMARY

Total sleep deprivation leads to decrements in neurobehavioral performance and changes in electroencephalographic (EEG) oscillations as well as the incidence of slow eye movements ad detected in the electro-oculogram (EOG) during wakefulness. Although total sleep deprivation is a powerful tool to investigate the association of EEG/EOG and neurobehavioral decrements, sleep loss during space flight is usual only partial. Furthermore exposure to the microgravity environment leads to changes changes in sodium and volume homeostasis and associated renal and cardio-endocrine responses. Some of these changes can be induced in head down tilt bedrst studies. We integrate research tools and research projects to enhance the fidelity of the simulated conditions of space flight which are characterized by complexity and mutual interactions. The effectiveness of countermeasures and physiologic mechanisms underlying neurobehavioral changes and renal-cardio endocrine changes are investigated in Project 3 of the Human Performance Team and Project 3 of the Cardiovascular Alterations Team respectively. Although the specific aims of these two projects are very different, they employ very similar research protocols. Thus, both projects investigate the effects of posture/bedrest and sleep deprivation (total or partial) on outcome measures relevant to their specific aims. The main aim of this enhancement grant is to exploit the similarities in research protocols by including the assessment of outcome variables relevant to the Renal-Cardio project in the research protocol of Project 3 of the Human Performance Team and by including the assessment of outcome variables relevant to the Quantitative EEG and Sleep Deprivation Project in the research protocols of Project 3 of the Cardiovascular Alterations team. In particular we will assess Neurobehavioral Function and Waking EEG in the research protocols of the renal-cardio endocrine project and renin-angiotensin and cardiac function in the research protocol of the Quantitative EEG and Waking Neurobehavioral Function project. This will allow us to investigate two additional specific aims:

1) Test the hypothesis that chronic partial sleep deprivation during a 17 day bed rest experiment results in deterioration of neurobehavioral function during waking and increases in EEG power density in the theta frequencies, especially in frontal areas of the brain, as well as the nonREM-REM cycle dependent modulation of heart-rate variability.

2) Test the hypothesis that acute total sleep deprivation modifies the circadian rhythm of the renin-angiotensin system, changes the acute responsiveness of this system to posture beyond what a microgravity environment alone does and affects the nonREM-REM cycle dependent modulation of heart-rate variability.

The data obtained on the waking EEG and neurobehavioral function in the chronic partial sleep deprivation experiment will complement the data obtained on the effects of total sleep deprivation which are collected in project 3 of the Human Performance Team. The data obtained on the renin-angiotensin levels in the acute total sleep deprivation experiment will complement data obtained on the effects of chronic partial sleep deprivation which will be collected in project 3 of the Cardiovascular Alterations team.

We have obtained recording in two subjects who participated in a 24 day laboratory study with 21 days of continuous bedrest. The application of identical research tools and outcome measures in research protocols across the Cardiovascular and Human Performance team will greatly enhance the overall science return of these projects and emphasizes the synergistic nature of this application.

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II. RESEARCH PLAN SUMMARY

A. HYPOTHESES, OBJECTIVES & SPECIFIC AIMS FROM ORIGINAL PROPOSAL

Exposure to the microgravity environment is associated with changes in multiple physiologic, endocrine and behavioral systems. These changes include sodium and volume homeostasis as well as sleep duration and structure. The consequences of these changes encompass a wide range of functions and the physiologic systems involved interact with each other. For instance, the renin-angiotensin system displays a profound modulation by sleep and the absence of sleep, i.e., sleep deprivation results in modification of the plasma levels of renin-angiotensin. Yet, these systems are often investigated in isolation. Nevertheless, the research tools applied to investigate the consequences of microgravity on both the volume regulating system and sleep are very similar, i.e., bedrest and sleep deprivation.

The main aim of this enhancement grant is to exploit the similarities in research protocols by including the assessment of outcome variables relevant to the Renal-Cardio project in the research protocol of Project 3 of the Human Performance Team and by including the assessment of outcome variables relevant to the Quantitative EEG and Sleep Deprivation Project in the research protocols of Project 3 of the Cardiovascular team. In particular, we will assess Neurobehavioral Function and Waking EEG as well as heart rate variability in the research protocols of the renal-cardio endocrine project and renin-angiotensin and cardiac function, i.e. heart-rate variability in the research protocol of the Quantitative EEG and Waking Neurobehavioral Function project. This will allow us to investigate two additional specific aims:

Additional Aim 1: Test the hypothesis that chronic partial sleep deprivation (i.e. 6.1 hours of sleep per night) during a 21 day bedrest experiment results in deterioration of neurobehavioral function during waking and increases in EEG power density in the theta frequencies, especially in frontal areas of the brain.

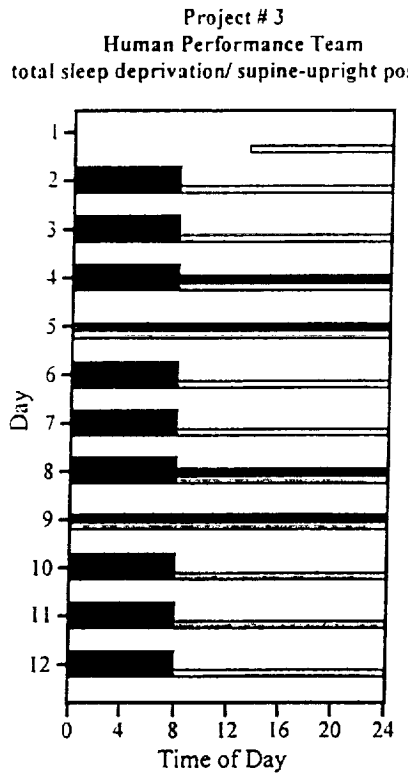
Additional Aim 2: Test the hypothesis that acute total sleep deprivation modifies the circadian rhythm of the renin-angiotensin system, changes the acute responsiveness of this system to posture beyond what a microgravity environment alone does and affects the nonREM-REM cycle dependent modulation of heart-rate variability.

The proposed integration of research tools and research projects provides a means to enhance the fidelity of the simulated conditions of space flight which are characterized by complexity and mutual interactions.

B. MODIFICATIONS REQUIRED UPON SELECTION

No modifications were required.

II. DETAILED RESEARCH PLAN-YEAR 1



A. Overview of Study Design Hypotheses

The purpose of the proposed studies is to test two specific hypotheses aimed at evaluating EEG based online monitoring as a practical and attainable tool to predict and prevent critical decrements in performance and alertness usually occurring after sleep curtailment typically encountered on shuttle missions and to investigated changes in the renin-angiotensin system associated with total sleep deprivation and changes in posture. These hypotheses are based partly on the results of our preliminary data (See Appendix B-3) which indicate that: (a) sleep loss comparable to sleep loss reported for spaceflight results in reduced subjective alertness and is also reflected in the spectral composition of the EEG during wakefulness. (b)

The two specific hypotheses are:

I) That chronic partial sleep deprivation during a 21 day bedrest experiment results in deterioration of neurobehavioral function during waking and increases in EEG power density in the theta frequencies, especially in frontal areas of the brain.

2) That acute total sleep deprivation modifies the circadian rhythm of the renin-angiotensin system, changes the acute responsiveness of this system to posture beyond what a microgravity environment alone does and affects the nonREM-REM cycle dependent modulation of heart-rate variability.

B. Specific Research Protocol

Experiment I

Experimental Subjects.

(This protocol is identical to Protocol I of Project 3 of the Human Performance Team)

Twelve healthy male and female volunteer subjects, aged 20-50, will be recruited for study. Only subjects who have provided written, informed consent for their participation in the study will be considered for study. Those volunteers who meet all of the screening criteria outlined below will be selected for study. All potential subjects will be required to maintain a regular sleep/wake schedule for two weeks prior to the start of study.

Experimental Procedure.

Ambulatory Baseline. The ambulatory baseline segment consists of 14 days, during which wrist activity and light levels will be recorded using ambulatory recording devices while the subjects are at home on a normal routine, maintaining a sleep log.

Baseline. The baseline segment begins with admission to the Intensive Physiologic Monitoring (IPM) Unit of the Brigham and Women's Hospital on Experimental Day 1 as illustrated in the figure and ends on the morning of Experimental Day 4. Physiologic, neurobehavioral EEG and ECG monitoring (as described in detail in the General

Methodology section below) commence upon admission on Experimental Day 1 and will continue throughout the duration of the study. Each day, subjects will be required to perform a battery of neurobehavioral tests every two waking hours. Subjects will continue to sleep and wake at their regularly scheduled times for three normal scheduled nights (solid black bars). Subject's sleep will be recorded polysomnographically during each Experimental Night.

Sleep Deprivation (40 h). There will be two sleep deprivation episode. The first 40 h sleep deprivation period will begin on the morning of Experimental day 4 and the second 40 h sleep deprivation will begin on the morning of Experimental day 8. During one sleep deprivation subjects will be in a supine posture while in the other sleep deprivation period subjects will be on a fixed schedule in which they alternate between sitting (20 min) and standing/walking (10 min). The order of these two sleep deprivation conditions will be randomized and balanced. At the end of each sleep deprivation episode blood pressure response to a change in posture will be measured according to the lying and standing blood pressure protocol. Blood will be sampled for melatonin and renin-angiotensin levels starting prior to the sleep episode preceding the sleep deprivation period and continue until the end of the sleep episode following the sleep deprivation period. The sampling frequency will be three samples per hour. EEG monitoring and ECG monitoring will be identical to the Baseline segment.

Recovery. The recovery segment will begin with sleep episode 4 and 7. Neurobehavioral, EEG and ECG monitoring will continue similar to the Baseline and Sleep Deprivation Segment. Subjects will be discharged upon awakening on Experimental Day 13.

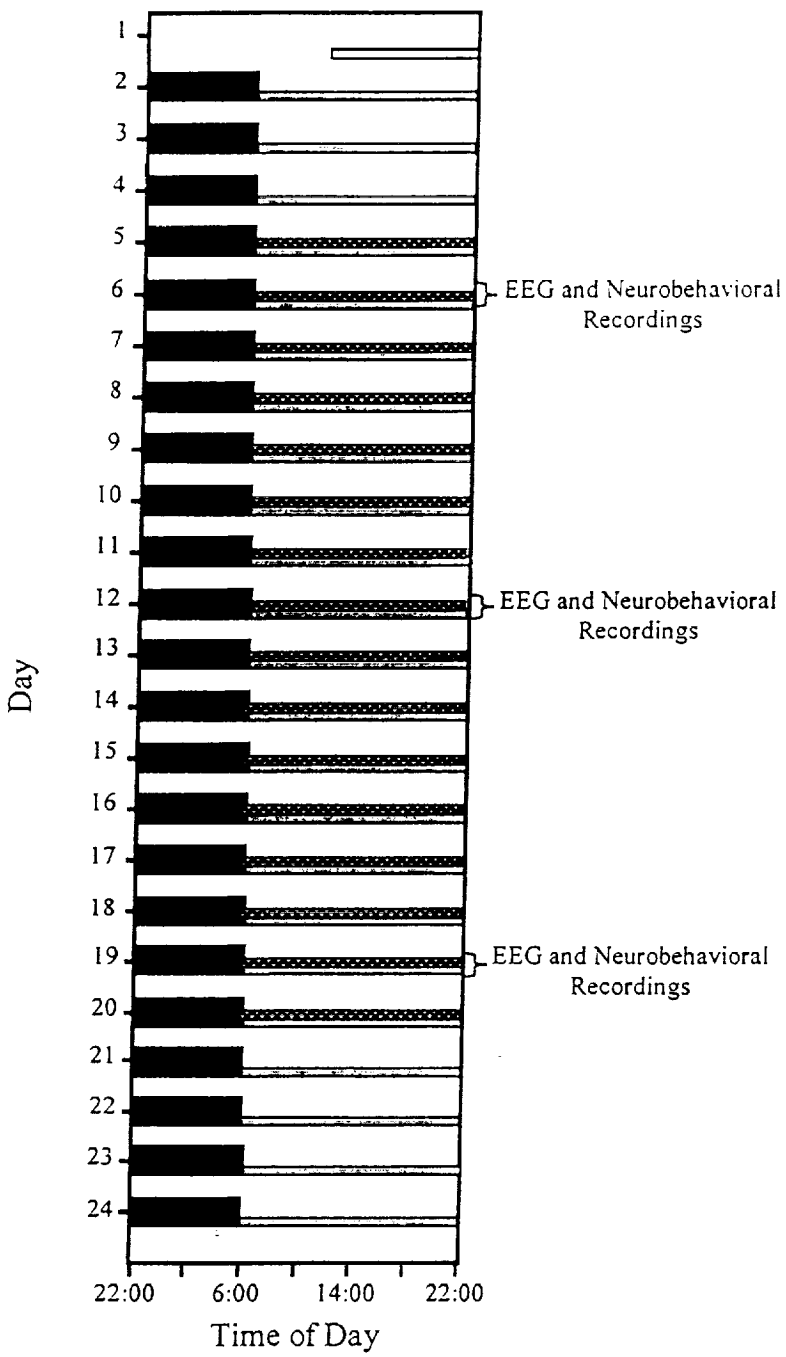
Experiment 2a and 2b

(These Protocols are Identical to Protocol I and IV of Project 3 of the Cardiovascular Team)

Rationale: Sleep deprivation and disruption of the relationship between sleep/wake cycle and the internal circadian rhythm produce substantial changes in the hormonal milieu and cardiovascular and renal functions Chronic partial sleep deprivation is common during space flight and results in changes in neurobehavioral function and the waking EEG.. However, the influence of chronic partial sleep deprivation in a continuous bedrest situation has not previously been investigated..

Experimental Subjects

Fourteen healthy volunteers (7 males and 7 females) will be recruited for the Experiment 2a (no sleep deprivation/bedrest) and 14 healthy volunteers (7 males and 7 females) will be recruited for Experiment 2b (partial sleep deprivation/bedrest). Details of their characteristics, recruitment and screening are provided in Appendix B of the Human Studies Core of Project 3 of the Cardiovascular Team)



Experimental Procedure The procedures will be as outlined in Human Studies Core of Project 3 of the Cardiovascular Alterations Team. In brief, after completion of the screening procedures, subjects will begin the first four phases of their study. Phase 1 will last four days and consists of equilibrating on a calculated, isocaloric, diet in the ambulatory center. During Phase 2 the subjects will be admitted to the Intensive Physiologic Monitoring (IPM) Unit of the GCRC, will maintain their diet, and will collect 24 hour urines daily for the duration of their hospital stay. Total volume, creatinine, sodium, and potassium will be measured daily with additional measurements at selected times (see EXPERIMENTAL TECHNIQUES). After two days of equilibration to the IPM Unit environment, a renal blood flow study will be performed. On the next morning beginning at 6:30 a.m., the acute perturbation studies will be performed. As described in the HUMAN STUDIES CORE, the acute interventions will be sequenced in the same order: LBNP, the posture study, followed by exercise. On completion of these three acute perturbations, the subjects will begin Phase 3: maintenance of bed-rest for 21 days. On the first day, a PAH clearance study will be performed in the morning. On the tenth day of bed-rest, a repeat PAH clearance will be performed. Phase 4 repeats the acute intervention studies and is identical to phase 2. They are repeated the first and fifth days after bed-rest In between testing, the subjects will be allowed ad-lib activity but continue their constant diet. Physiologic, neurobehavioral, EEG monitoring and ECG monitoring commence upon admission on Experimental Day 1 and will continue throughout the duration of the study. Each day, subjects will be required to perform a battery of neurobehavioral tests every two waking hours. Subjects will continue to sleep and wake at their regularly scheduled times for three normal scheduled nights (solid black bars). Subject's sleep will be recorded polysomnographically during each Experimental Night.

Experimental Design of experiment 2b: There are four phases to this protocol. Phases 1, 2, and 4 will be identical to that outlined under Specific Aims 1 and 2. Phase 3 is modified. The microgravity environment will be maintained, but the subjects will be in their dark cycle for only 6.1 hours rather than 8 hours. This will occur by maintaining their waking time at 6:30 a.m. but delaying the dark cycle start until 12:24 a.m. Thus, they will be in a microgravity, sleep-deprived environment. As in Specific Aim 1, acute responsiveness of the RAAS will be one primary end point. The second added end point will be neurobehaviroal performance and the waking EEG.

III. PROGRESS, RESULTS & ACCOMPLISHMENTS

This synergy grant was awarded in April of 1998. The progress that has since been made includes finalizing the details of the experimental design and initializing the purchase of the VITAPORT digital sleep recorder. In particular the bed rest protocol was extended so that the total duration of the protocol is now 24 days. We successfully recorded more than 100 h of EOG and EEG in two volunteers in the 8-h sleep condition (continuous bed rest with head down tilt) while these subjects completed neurobehavioral testing at scheduled intervals. These data sets are currently being analyzed to investigate whether the associations between EOG/EEG and neurobehavioral performance which we observed during our research in protocol 3, are also present during continuous bed rest.

The ambient lighting conditions for one subject are illustrated in Figure1. The light

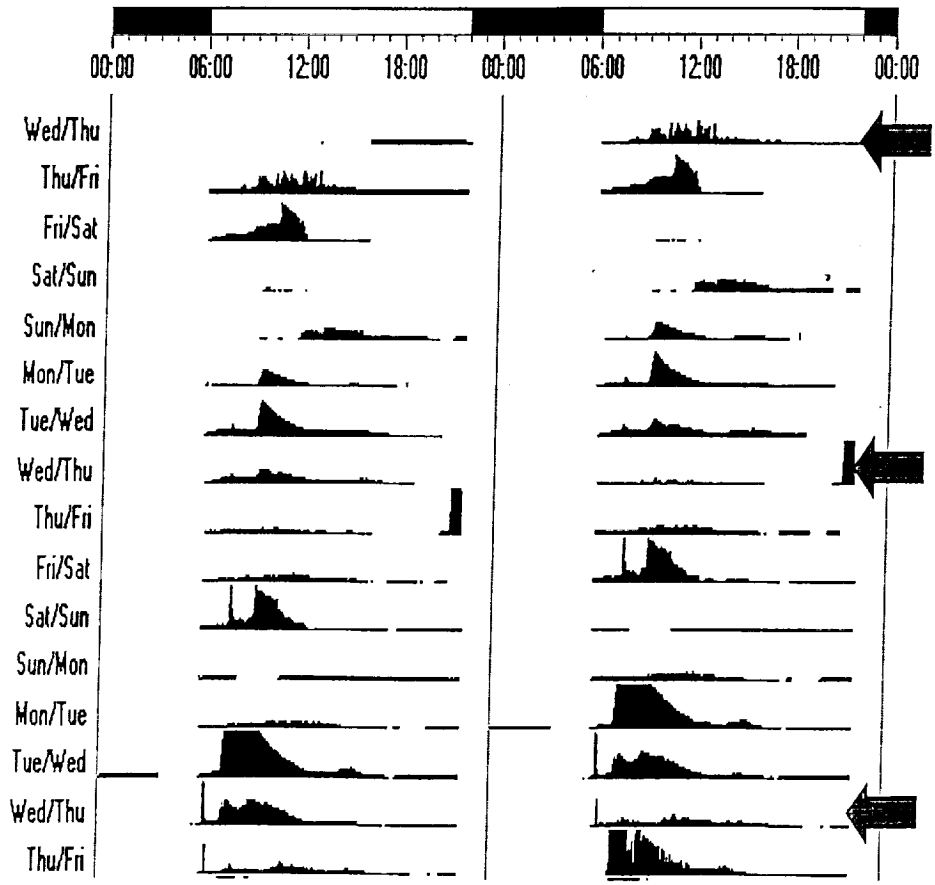


Figure 1: douple plot of room light levels (lux). Black bars on the top idicate where the lights were out (22:00 to 6:00 h) and grey arrows the days when the EEG was recorded in this subject.

levels were measured with an ActillumeLwatch mounted on the wall behind his head. Lights out times were scheduled between 22:00 and 6:00 am. During this interval the subject was allowed to sleep. The arrows on the left indicate the days when the EEG was recorded continuously. The day to day variability in daytime light levels was rather large (see Table1) because the subject's room had a window and shades. Interstingly, illuminance in this room was highest in the morning hours from 6 am to noon.

Table 1: Mean and maximum light intensity (lux) between 6 am and 10 pm from bedrest day 2 to 16 of the experiment (ActillumeLwatch).

Bedrest day	Mean	Max
2	41	275
3	42	478
4	5	13
5	22	135
6	21	223
7	46	442
8	32	192
9	22	600
10	18	104
11	55	965
12	7	16
13	17	87
14	115	1192
15	56	557
16	25	353
Mean	34.9	375.5
Stderr	(27.2)	(343.9)

For the same subject the incidence of slow eye movements (SEMS) during the indicated days was assesed from two electro-oculogram derivations. For every 30-s epoch througout the scheduled wake episode a scorer decided whether or not at least one SEM occurred (Figure 2). The incidence of SEMS was high after lights on at 6 am in the morning. In general, the levels of SEMS were high particularly during the first and third recording, where a 3-h episode with a lot of SEMS and even sleep was observed. This may indicate that subjects under continuous head down bedrest conditions are less alert than subjects under strict controlled constant routine conditions or even real life settings. We are currently analyzing the association between SEMs and neurobehavioral performance assessments.

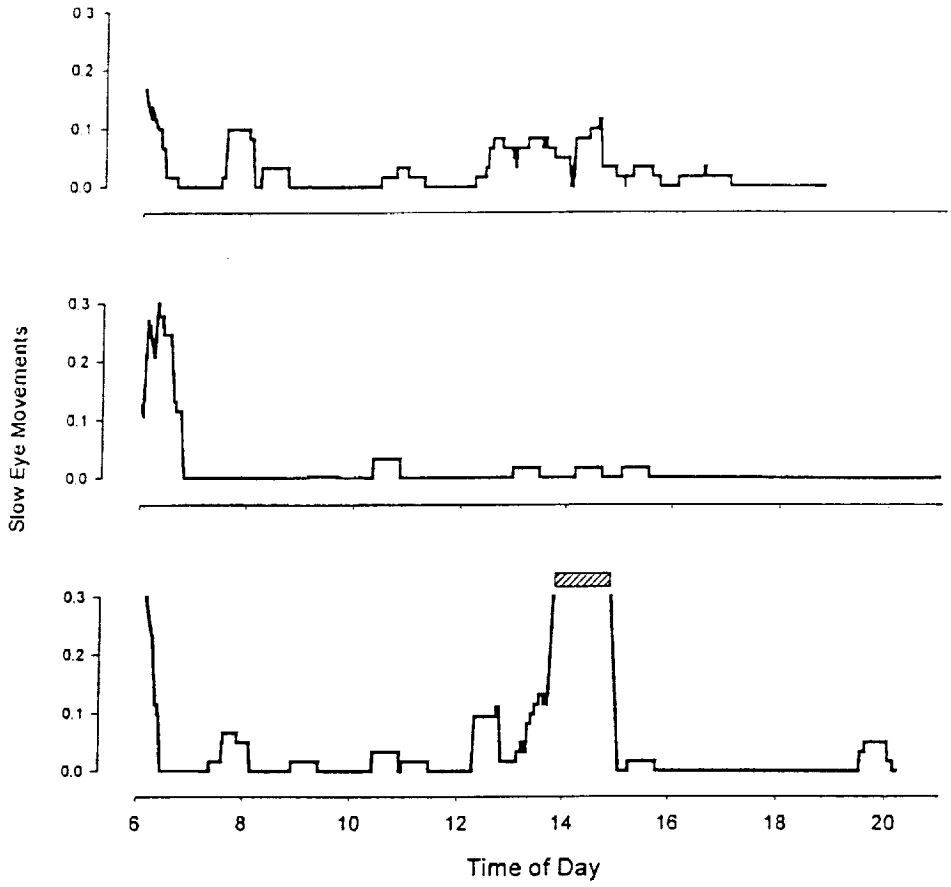


Figure 2: Incidence of SEMS recorded in one subject during the 2 the, 9 th and 16 th bedredst day from 6am to 20:30h. Hatched rectangluar area indicates episode of sleep (nap).

IV. IMPLICATIONS FOR FUTURE RESEARCH

We plan to continue the synergy research in year 3 of our grant and expect to obtain EEG/EOG recordings both during the 8-h bedrest condition as well as during the 6.1 h condition. The latter condition will allow us to investigate the association between EEG/EOG and neurobehavioral performance in sleep restriction conditions that are to some extent similar to those in micro-gravity.

APPENDIX A.

Publications supported by this grant

None.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

1999 Annual Project Report

Research Teams: Cardiovascular Alterations and Neurovestibular Adaptation

Project Name: Visual- and Vestibular-Autonomic Influence on Short-term Cardiovascular Regulatory Mechanisms

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EXECUTIVE SUMMARY

This synergy project was a one-year effort conducted cooperatively by members of the NSBRI Cardiovascular Alterations and Neurovestibular Adaptation Teams in collaboration with NASA-Johnson Space Center (JSC) colleagues. The objective of this study was to evaluate visual-autonomic interactions on short-term cardiovascular regulatory mechanisms. Based on established visual-vestibular and vestibular-autonomic shared neural pathways, we hypothesized that visually induced changes in orientation will trigger autonomic cardiovascular reflexes. A second objective was to compare baroreflex changes during postural changes as measured with the new Cardiovascular System Identification (CSI) technique with those measured using a neck barocuff. While the neck barocuff stimulates only the carotid baroreceptors, CSI provides a measure of overall baroreflex responsiveness.

This study involved a repeated measures design with 16 healthy human subjects (8 M, 8 F) to examine cardiovascular regulatory responses during actual and virtual head-upright tilts. Baroreflex sensitivity was first evaluated with subjects in supine and upright positions during actual tilt-table testing using both neck barocuff and CSI methods. The responses to actual tilts during this first session were then compared to responses during visually induced tilt and/or rotation obtained during a second session.

Effect of actual changes in posture on baroreflex responses. CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate (HR), arterial blood pressure (ABP), and instantaneous lung volume (ILV, respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. A random interval breathing protocol (mean rate of 12 breaths per minute, inter-breath intervals randomly varying between one and 15 seconds) is utilized to broaden the frequency content of the recorded physiological signals, thereby facilitating CSI. Using the CSI technique, we have previously observed significant alterations to the autonomically mediated coupling mechanisms with a change in posture from supine to upright, while non-autonomically mediated mechanisms are left essentially unchanged. Further analysis of data from this first session will utilize CSI measurements to confirm this result, and to quantitatively compare the neck barocuff method with CSI in estimating baroreflex sensitivity.

Carotid baroreflex responses were obtained in both supine and head upright tilt positions using the neck barocuff employed according to the method described by Fritsch *et al.* (1992). This technique allows assessment of vagally mediated carotid baroreceptor-cardiac reflex responses provoked by neck pressure and suction steps during held expiration. Pressure was increased to 40 mmHg for 5 seconds, reduced by 15 mmHg decrements after each of the next seven R waves to -65 mmHg, and finally returned to ambient levels. Responses from up to four successful repetitions of this stimulus sequence during both supine and upright positions were averaged. R-R intervals were plotted against carotid distending pressure (taken to be systolic minus neck chamber pressures). There were significant differences between male and female subjects for both minimum and maximum RR interval ($p<0.01$). For both male and female subjects, there were highly significant decreases ($p<0.0001$) in minimum and maximum RR intervals when subjects were tilted from the supine to upright position. There were not significant differences in either the RR interval ranges or maximum slopes between these positions.

Cardiovascular responses during virtual tilt and/or rotation. A second session with the same subjects was then used to examine the effects of visually induced virtual tilt and/or rotation stimuli in modulating autonomic cardiovascular reflexes. One of the stimuli involved a simple "mirror bed" to provide an illusion of body tilt without rotation. This device involved mounting a mirror over a subject in a supine orientation to align surrounding visual vertical cues with the subject's longitudinal body axis. In addition to the mirror bed, visually induced tilt and/or

rotation illusions were elicited by a full-field virtual environment generator at NASA known as the Preflight Adaptation Trainer DOME. The subject was supine with the head positioned near the center of this large spherical DOME, and a virtual scene aligned with the longitudinal body axis was then rotated in pitch, yaw or roll planes to elicit sensations of tilt and/or rotation.

The conditions were chosen to provide the following combinations of perceived tilt and/or rotation:

Mirror bed – perceived tilt without rotation
DOME Pitch and Yaw – perceived tilt and rotation
DOME Roll – perceived rotation without tilt

Although there was a high degree of variability across subjects, the mean responses reflect the expected combinations of perceived tilt and rotation described above. The mirror bed was rated by subjects to be the most compelling, with the perceived orientation of the head (54.7±6.7, mean ±SEM) slightly greater than the perceived orientation of the body (45.0±5.7). Cardiovascular responses were recorded during 2 min prior to the start of each virtual tilt and during the initial three minutes with eyes open. Although the data appear to be quite variable, there were a few instances when the changes were quite dramatic. For example, rapid decreases in both systolic and diastolic pressure were observed in some subjects at the onset of the virtual tilt similar to the changes in blood pressure to an actual change in body posture on a tilt table.

Our preliminary results suggest that visually induced virtual tilt can elicit at least transient cardiovascular changes in some individuals. Pending further analysis, we expect to find that the degree of change in cardiovascular reflexes will correlate with individual measures of tilt perception. We will further characterize these effects on cardiovascular regulatory mechanisms using CSI, and expect that visually induced tilts will result in reductions in HR baroreflex sensitivity. The significance of these findings is that virtual environment stimuli may be used in the future to enhance cardiovascular and/or vestibular countermeasures for long-duration spaceflight.

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I. RESEARCH PLAN SUMMARY

A. Hypotheses, Objectives & Specific Aims From Original Proposal

This synergy project was a one-year effort conducted cooperatively by members of the NSBRI Cardiovascular Alterations and Neurovestibular Adaptation Teams in collaboration with NASA-Johnson Space Center (JSC) colleagues.

It has been well established that visual and vestibular sensory input are integrated in the central nervous system to provide information about changes in orientation and motion (e.g., Robinson, 1977). Static visual cues, for example, are sufficient to elicit changes in the perception of verticality, while a moving visual scene results in an illusion of self-rotation (Howard, 1982). Substantial evidence has also accumulated in recent decades of anatomic connections between vestibular and autonomic nuclei in animals (Yates *et al*, 1995; Yates 1992; Yates *et al.*, 1993, Yates *et al.*, 1994). Although direct evidence in human subjects remains limited, it is becoming increasingly clear that the vestibular system may exert significant influence on autonomic cardiovascular reflexes (Biaggioni et al, 1998).

Blunting of the carotid-cardiac baroreflex has been observed during and after spaceflight and prolonged bed rest using the neck barocuff technique (Fritsch *et al.*, 1992; Fritsch-Yelle *et al.*, 1994). This impairment in baroreflex mechanisms may contribute to orthostatic intolerance observed in astronauts upon return to earth. It has been suggested that vestibular pathways could play a significant role in changing the orthostatic competence of astronauts by inhibition of vagal withdrawal and elevation of parasympathetic outflow. However, functional interpretation of neck barocuff data is complicated because this method stimulates only the carotid baroreceptors. Interpretation would be more straightforward if blunting could be demonstrated using a method like Cardiovascular System Identification (CSI) which provides a measure of overall baroreflex responsiveness.

The general objectives of the study were (1) to evaluate, in human subjects, the roles of visual and vestibular systems in modulating autonomic cardiovascular reflexes, and (2) to quantitatively compare the neck barocuff method with Cardiovascular System Identification (CSI) in estimating baroreflex sensitivity. Specifically, we planned to test the following hypotheses:

1. That visually induced illusions of a change in perceived orientation with respect to gravity and/or visually induced illusions of rotation will evoke a change in autonomic modulation of cardiovascular reflexes as measured by CSI and the neck barocuff technique.

2. That vestibular stimulation induced by true rotation in yaw modulates the HR baroreflex gain as measured by CSI and the neck barocuff technique.

3. That CSI estimates of HR Baroreflex sensitivity correlate with those determined by the neck barocuff technique.

B. Modifications Required & Rationale For Modifications.

Our original protocol included one session to assess baroreflex sensitivity using two methods (cardiovascular system identification and neck barocuff) with subjects in supine and upright positions on a tilt table and using a virtual environment generator (VEG) to elicit tilt illusions. In order to simplify the protocol and minimize the effects of the neck barocuff on the perception of tilt, we elected to split this session into two parts using a repeated measures design as follows.

During the initial session, baroreflex sensitivity was evaluated with subjects in supine and upright positions during actual tilt-table testing using both CSI and neck barocuff techniques. During this session we were able to accomplish one of our primary aims, namely to quantitatively compare the neck barocuff method with Cardiovascular System Identification (CSI) in estimating baroreflex sensitivity.

A second session with the same subjects was then used to complete our second objective to examine the effects of visually induced tilt and/or rotation in modulating autonomic cardiovascular reflexes. Baroreflex sensitivity was measured during this second session using only the CSI technique. One original objective to duplicate a study by Convertino *et al* .(1997) examining carotid baroreflex responsiveness during sinusoidal rotation in yaw was deleted in order to focus on visual-autonomic interactions. The main rationale for this was to present all visual stimuli with subjects in a supine orientation, thus avoiding stimulation of non-vestibular gravireceptors (e.g. baroreceptors).

Another modification of the study was the use of alternate stimuli to visually induce illusions of tilt and/or rotation. The original proposal included continuous scene motion to be provided by NASA's head-mounted VEG. However, as part of another NSBRI investigation (Visual Orientation in Unifamiliar Gravito-Intertial Environments, PI Charles Oman), Ian Howard at York University devised a simple "mirror bed" to provide an illusion of body tilt while in a supine orientation. This concept involves mounting a mirror over the subject to align surrounding visual vertical cues with the subject's longitudinal body axis. One advantage of this technique is that it elicits a strong illusion of tilt without scene motion. Based on the encouraging preliminary results from Howard's laboratory, we duplicated the mirror bed for data collection in the JSC Neuroscience Laboratory. Virtual scene rotation conditions were also retained during this second session. However, rather than using the head-mounted VEG, a full-field virtual scene was provided by NASA's Preflight Adaptation Trainer DOME. The virtual scene was aligned with the longitudinal body axis and then rotated in pitch, yaw and roll planes to elicit sensations of tilt and/or rotation.

C. Summary Of Progress In Year 1.

This Synergy project was selected for funding during the first year (April, 1998). As described below, this one-year project has been carried out during Year 2.

D. Actions Taken In Response To Last Year's Critique, If Any.

No additional modifications or actions were required in response to last year's NSBRI critique.

II. DETAILED RESEARCH PLAN – YEAR 2

This study involved a repeated measures design to examine cardiovascular regulatory responses to actual and virtual head-upright tilts. During the first session, baroreflex sensitivity was evaluated with subjects in supine and upright positions during actual tilt-table testing using both neck barocuff and Cardiovascular System Identification (CSI) methods. The responses to actual tilts during the first session were then compared to responses during visually induced tilt and/or rotation obtained during the second session.

Sixteen healthy, normotensive, non-smoking human subjects (8 male, 8 female, ages 20-50 yr.) participated in both sessions. An additional female subject participated in the first session only. Subjects were asked to refrain from consuming caffeine or performing rigorous exercise for 24 hours prior to each session of the study. A 20 min rest period in a supine position to allow for hemodynamic equilibration preceded each data collection period.

Session 1: Actual Tilt Table Testing

During the first session, the CSI measurements were obtained during 8 min of random interval breathing in the supine position just prior to tilt, and then following 5 min of 80 deg head upright tilt. The neck barocuff responses were also obtained in the supine position and after 5 min of head upright tilt. The order of neck barocuff and CSI was counterbalanced across subjects, with a 20 min rest period in the supine position between measures. Transient responses during the first three minutes of tilt and following return to the supine position were obtained with some subjects for comparison with the visually-induced tilt conditions during the second session.

Cardiovascular System Identification (CSI). CSI involves the mathematical analysis of second-to-second fluctuations in non-invasively measured heart rate (HR), arterial blood pressure (ABP), and instantaneous lung volume (ILV, respiratory activity) in order to characterize quantitatively the physiologic mechanisms responsible for the couplings between these signals. Through the characterization of all the physiologic mechanisms coupling these signals, CSI provides a model of the closed-loop cardiovascular regulatory state in an individual subject. The model includes quantitative descriptions of the heart rate baroreflex as well as other important physiologic mechanisms. In order to completely identify the CSI model, eight minute segments of ECG, ILV, and ABP were obtained. In addition to the continuous measurements obtained from a finger cuff device (Finapresss), blood pressure was also measured with an automatic oscillometric device (Dinamap) before and after each CSI period. ILV was measured using a non-invasive Respitrace system (Ambulatory Monitoring Systems, Inc.) which was also calibrated before and after each CSI period. Data were collected using a random interval breathing protocol (Berger et al., 1989) during which subjects breathe in response to auditory cues at a comfortable mean rate of 12 breaths per minute but with inter-breath intervals randomly varying between one and 15 seconds. Subjects adjust their own tidal volumes thereby leaving blood gases unperturbed. The random interval breathing protocol broadens the frequency content of the recorded physiological signals, thereby facilitating CSI.

During this first session, Ultrasound Doppler measurements of aortic flow were also made during CSI from a transducer focused from the suprasternal notch. This beat-to-beat measure of stroke volume will be used for an extended CSI model analysis. Analyzing the fluctuations between stroke volume with either the preceding R-R interval, or the succeeding arterial pressure pulse should allow quantitative estimates of ventricular contractility, ventricular compliance, and the peripheral resistance baroreflex. (Doppler was not obtained during the neck barocuff or during CSI measurements in the second session to avoid disturbing the perceptual illusions with the hand-held probe.)

Neck barocuff. The neck barocuff was employed according to the method described by Fritsch *et al.* (1992). A tightly sealing Silastic chamber was strapped to the anterior neck. A computer controlled stepping-motor driven bellows delivered a fixed sequence of neck pressure and suction steps to the chamber during held expiration, as follows. Pressure was increased to 40 mmHg for 5 cardiac cycles, reduced by 15 mmHg decrements after each of the next seven R waves to -65 mmHg, and finally returned to ambient levels. Responses from up to four successful repetitions of this stimulus sequence during both supine and upright positions were averaged. R-R intervals were plotted against Carotid distending pressure (taken to be systolic minus neck chamber pressures). Minimum, maximum and range of RR intervals, and the maximum slope were obtained for each subject per condition.



Figure 1. Subject in upright position on tilt table is instrumented with Finapress, ECG and Respitrace for ABP, HR and ILV measurements, respectively. The neck barocuff shown here was utilized to measure baroreflex responses in both supine and upright positions.

Session 2: Virtual Tilt and/or Rotation Testing

During the second session, baseline CSI measurements were obtained during random interval breathing in the supine position following a 20 min supine rest period. The baseline CSI measures were obtained with the subjects' eyes closed while lying supine on either the mirror bed or inside the DOME, whichever stimulus came first. All 16 subjects were exposed to the mirror bed tilt stimulus. Equipment problems with the DOME limited the number of subjects exposed to the virtual scene rotation to 7 of 18 subjects. The test order was counterbalanced for subjects exposed to both types of stimuli.

Mirror bed. The mirror bed consisted of a tall bed with a thick foam cushion, adjustable footrest and mirror attachment (Figure 3). The bed was raised to four feet tall to have subjects view the surrounding room at a height as close to normal eye level as feasible. The mirror was made of plexiglass mounted to a plywood sheet for structural support. The mirror pivoted to a 45 deg angle in front of the subject's face so that room vertical cues are aligned with the longitudinal body axis, and the bottom edge of the mirror was contoured to increase the field-of-view. The bed was positioned so that the subjects viewed instrument consoles and a doorway to provide strong vertical polarity cues. After positioning the subject on the bed relative to the mirror, a footrest was clamped in place to provide a tactile sense of a floor underneath and to control for feet orientation. Care was taken to avoid applying pressure with the footrest to minimize muscle pump activity in the lower limbs. After the initial setup, subjects completed their rest and baseline CSI measurements with eyes closed.

Figure 2 below illustrates the mirror bed protocol and provides the mean responses for the tilt perception. Cardiovascular parameters were recorded for 2 min prior to asking the subjects to open their eyes, and transient responses were obtained during the first 3 min of the virtual tilt. Subjects were then asked to verbally report their perception of body tilt position in degrees, with 0 deg equal to no tilt and 90 deg equal to standing upright. Separate measures were obtained for the perception of head orientation. After obtaining the initial tilt perception following 3 min of exposure, the Respitrace system was calibrated by having subjects breath into a 800 cc bag, and the random interval breathing was performed for 8 min. Following the CSI measurements, a second report of tilt perception for body and head orientation was obtained (after approx. 13 min elapsed time), and a second Respitrace calibration was performed. Cardiovascular parameters were then obtained just prior to and after the subjects closed their eyes to assess after-effects of the tilt illusion. A final measure of tilt perception was obtained following 3 min with the subject's eyes closed.

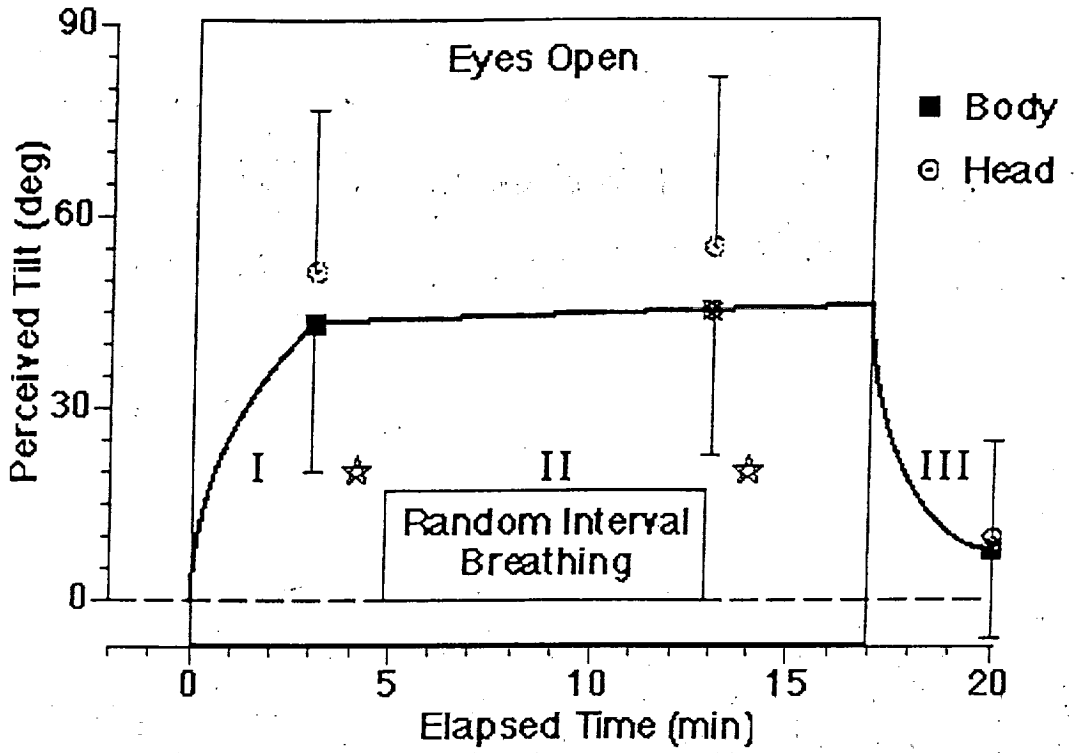


Figure 2. Cardiovascular parameters were obtained during three data collection periods on the mirror bed: transient changes during the first 3 min (I), CSI measurements between 5 – 13 min (II), and transient changes for 3 min following eyes closed (III). Star symbols indicate when Respitrace calibrations were obtained before and after the random interval breathing period. Perception of body and head tilt orientation were obtained after 3 and 13 min with eyes open, and after 3 min with eyes closed. Symbols depict means ± standard deviations (n=18). The onset and decay of tilt perception shown are illustrative of drawings obtained from subjects to describe the time course of tilt perception.

DOME. The JSC Neuroscience Laboratory's Device for Orientation and Motion Environments (DOME) was used during the second session with 7 subjects to provide a virtual room environment eliciting the sensation of tilt and/or rotation while lying supine. The subject was supine with the head positioned near the center of the 12-foot diameter spherical dome (Figure 3). Subject lied on the same foam cushion as used on the mirror bed, and a footrest was also

utilized. Two video projectors displayed a wide-angle view of video images on the top interior of the dome. The images consisted of a checkerboard virtual room with vertical cues (doorway, stick figure, window, signs) aligned with the longitudinal body axis.

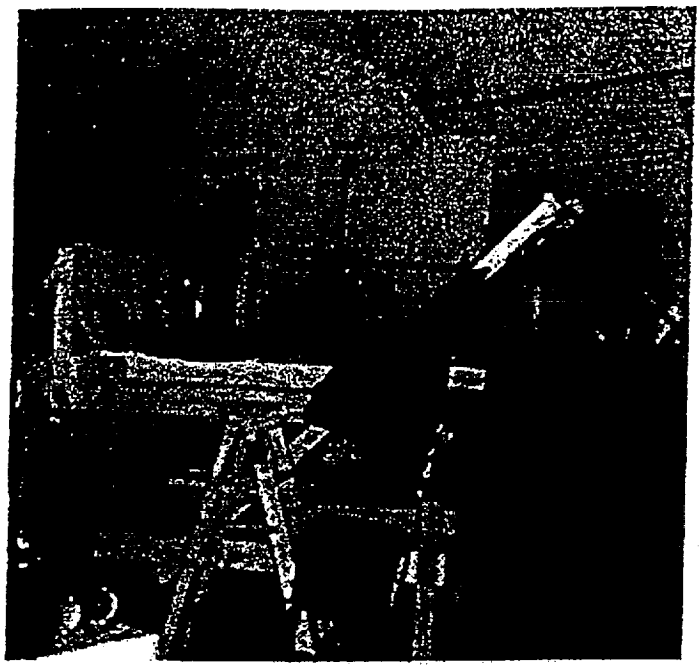


Figure 3. Subject lies supine on the mirror bed to elicit the sensation of tilt. The DOME shown in the background was used to provide virtual scene movement to elicit the sensation of tilt and/or rotation.

During the virtual scene presentation, the door was closed and the cardiovascular equipment was located on the adjoining support platform. Perceptual reports and the audio tones for the random interval breathing were provided over a two-way audio communication system. After the 20-minute rest period, the virtual scene was rotated in either the subject's pitch, yaw or roll plane at 35 deg/sec. The protocol sequence was similar to that described above for the mirror bed. Perception reports of body tilt orientation and percent self-motion were obtained after 3 min and 13 min of exposure to the virtual scene motion, with the CSI measurements obtained inbetween. Experimenters entered the virtual scene for the Respitrace calibration just prior to the CSI measurements, and this resulted in a brief disruption of the perception of tilt and/or rotation. However, most subjects reported that this was only temporary, with a quick recovery as soon as the DOME door was closed. There was a 10 min rest period inbetween DOME stimuli, and the order was counterbalanced across subjects.

Following the exposure to either the mirror bed or DOME stimuli, subjects were asked to use a visual analog scale to judge the certainty of the perceived tilt by indicating how the illusion of tilt compared to an actual tilt (0 = "Not at all the same", 100 = "Exactly the same"). Subjects also rated the relative compellingness of the mirror bed and the different DOME stimuli. A visual analog scale was also used to report the constancy of the perceived tilt (0 = "Was transient", 100 = "Never changed").

III. PROGRESS, RESULTS & ACCOMPLISHMENTS – YEAR 2

During this year, the modifications to the experiment protocol described above were approved by the JSC institutional review and ground safety boards. The mirror bed was fabricated based on the design specifications provided by Ian Howard's laboratory at York University. At this time, the CSI data has not been delivered from JSC for final processing. The preliminary results from the neck barocuff are described below for the actual tilt table test session. Perceptual data and a sample of a transient response to the mirror tilt stimulus demonstrate the preliminary findings from the virtual tilt test session.

Session 1: Actual Tilt Table Testing

During the first session, carotid baroreflex responses were obtained in both supine and head upright tilt positions. A repeated measures analysis of variance (ANOVA) was performed with body position as the dependent (within) variable and gender as the between factor. As illustrated in Figure 4, there were significant differences between male and female subjects for both minimum and maximum RR interval ($p<0.01$). Although the male subjects generally had greater RR interval ranges and greater maximum slopes, these differences were not significant at the 0.05 level .

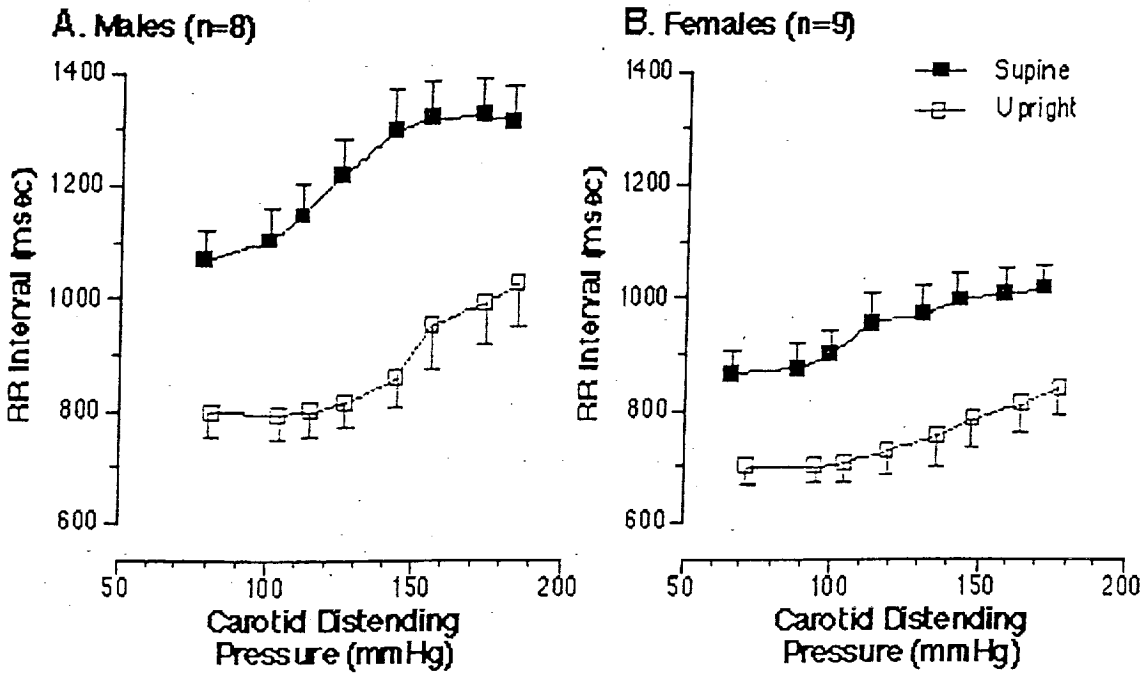


Figure 4. Neck barocuff responses (mean ± stand deviation) for males (A) and females (B) for both supine (closed squares) and upright (open squares) positions. Error bars represent standard error of means (SEM).

For both male and female subjects, there were highly significant decreases ($p<0.0001$) in minimum, maximum and control RR intervals when subjects were tilted from the supine to upright position. There were not significant differences in either the RR interval ranges or maximum slopes between these positions.

Using the CSI technique, Mullen et al. (1997) had previously noted that a change in posture from supine to upright results in significant alterations to the autonomically mediated coupling

mechanisms while leaving non-autonomically mediated mechanisms essentially unchanged. Further analysis of data from the first session will utilize CSI measurements from the random breathing intervals to confirm this result, and to quantitatively compare the neck barocuff method with CSI in estimating baroreflex sensitivity.

Session 2: Virtual Tilt and/or Rotation Testing

The four virtual stimuli used in the second session allowed us to evaluate the effects of visually-induced tilt and/or rotation on autonomic regulatory mechanisms. The conditions were chosen to provide the following combinations of perceived tilt and/or rotation:

Mirror bed – perceived tilt without rotation
DOME Pitch and Yaw – perceived tilt and rotation
DOME Roll – perceived rotation without tilt

The following table provides the mean responses (± standard errors) for each stimulus after approximately 3 and 13 min exposure to each stimulus. Although there was a high degree of variability across subjects, the mean responses reflect the expected combinations of perceived tilt and rotation as described above. Figure 5 illustrates the distribution of perceived tilt orientation for each stimulus after 3 min exposure.

Table 1. Mean responses from verbal reports.

Stimulus	Perceived Tilt Orientation		Perceived Self-motion	
	3 min	13 min	3 min	13 min
Mirror bed			N/A	N/A
- body	42.8 (±5.7)	45.0 (±5.7)		
- head	51.1 (±6.4)	54.7 (±6.7)		
DOME Pitch	23.8 (±13.9)	13.1 (±13.7)	74.4 (±11.0)	55.6 (±14.9)
DOME Yaw	15.7 (±15.8)	14.3 (±15.6)	65.7 (±14.8)	55.7 (±16.3)
DOME Roll	0.0 (±19.6)	1.4 (±19.6)	66.4 (±13.7)	47.9 (±14.6)

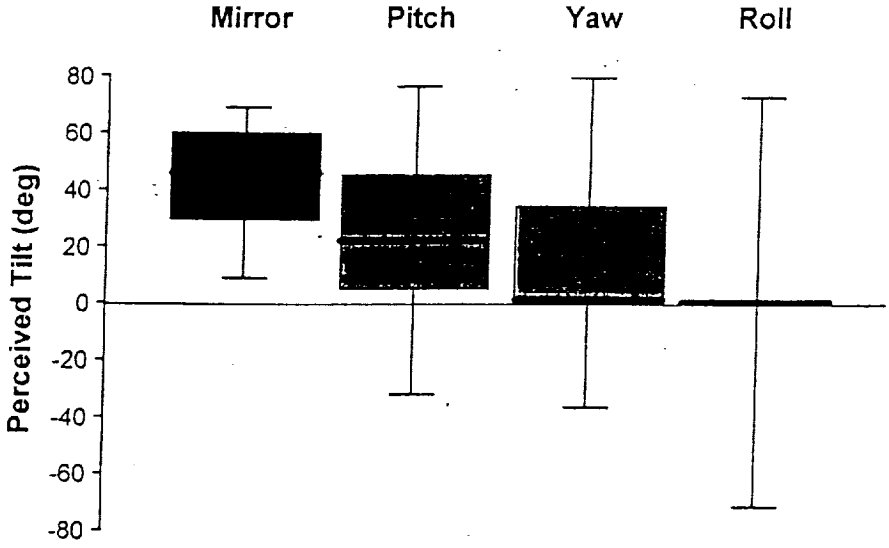


Figure 5. The distribution of perceived tilt orientation for each virtual stimulus after 3 min exposure. The box plots show the 10th, 25th, 50th (median, heavy line), 75th and 90th percentiles.

As illustrated in Figure 2 and Table 1 above, the perceived orientation of the head was slightly greater than the perceived orientation of the body on the mirror bed. Although there was a trend for the tilt illusion to become stronger over time on the mirror bed and slightly weaker during the DOME stimuli, these differences were not statistically significant. The percent of perceived self-motion with the DOME stimuli also had a tendency to decrease over time, and therefore it is likely that there were more dropouts in perception with scene movement in the DOME versus the static visual scene with the mirror. The relative rating of how compelling the sensation of tilt was for the different stimuli followed the same pattern as in Figure 5: Mirror > Pitch > Yaw > Roll. The certainty visual analog scale measures also indicated that the mirror bed was generally the most compelling virtual tilt stimulus.

Transient responses during virtual tilt onset. The cardiovascular responses were recorded during 2 min prior to the start of each virtual tilt and during the initial three minutes with eyes open. Although this data analysis is still in progress, there were a few instances when the changes were quite dramatic. One of the best examples of this is shown in Figure 6. The trace clearly shows a rapid decrease in both systolic and diastolic pressure at the onset of the virtual tilt similar to the changes in blood pressure to an actual change in body posture on a tilt table. As with the perceptual data, there appears to be a high degree of variability in transient responses to the virtual tilt stimuli. Future analysis will focus on correlating the magnitude of perceived tilt with the magnitude of cardiorespiratory changes.

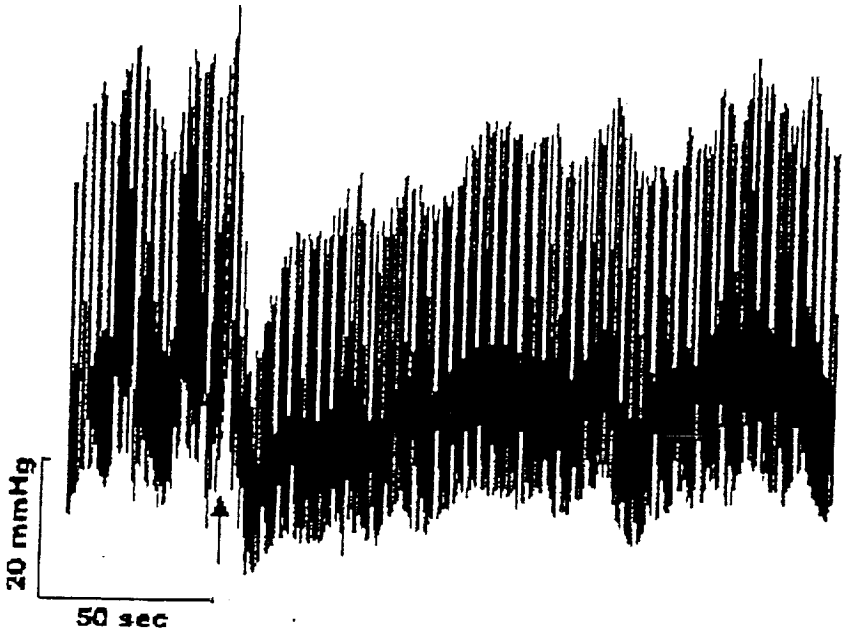


Figure 6. Changes in blood pressure (obtained from Finapress) at the onset of the mirror tilt stimulus. The upward arrow indicates when the subject eyes are opened.

IV. IMPLICATIONS FOR FUTURE RESEARCH & PLAN FOR YEAR 3

Our preliminary results suggest that visually-induced virtual tilt can elicit at least transient cardiovascular changes in some individuals. Pending further analysis, we expect to find that the degree of change in cardiovascular reflexes will correlate with individual measures of tilt perception. We will further characterize these effects on cardiovascular regulatory mechanisms using CSI, and expect that visually induced tilts will result in reductions in HR baroreflex sensitivity.

Our study provides quantitative data for evaluating the relationship between neck barocuff and CSI assessments of baroreflex sensitivity. If CSI is determined to offer assessments of baroreflex sensitivity equivalent or superior to those of the neck barocuff technique, it may prove to be a more cost effective and less invasive alternative for ground-based and, more importantly, for flight research. In cooperation with the Cardiovascular Alterations and Technology Development Teams, we are continuing to develop improved algorithms for Cardiovascular System Identification. For example, we expect that the additional stroke volume data we obtained with the Doppler technique during the actual tilt-table testing will specifically be used to develop enhanced measures of ventricular contractility, ventricular compliance, and the peripheral resistance baroreflex. We are also working closely with the Technology Development Team on an automated acquisition and analysis system.

Although this synergy project is ending, the results have promising implications for future research. First, the apparent high degree of variability with responses to visually-induced tilts should not be that surprising given the variability with other vestibular-autonomic interactions (e.g. motion sickness susceptibility). One area of interest would be to utilize these individual differences and characterize visual field dependence across subjects, e.g. using a rod and frame test, and correlate these measures with the cardiovascular responses to virtual tilt stimuli. It would also be interesting to test labyrinthine deficient patients with similar virtual tilt stimuli as used in our study. Finally, one of the most intriguing aspects for new research is the potential for using virtual environment stimuli to enhance future cardiovascular and/or vestibular countermeasures for long-duration spaceflight.

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APPENDIX A. Publications supported through NSBRI funding

Wood SJ, Ramsdell CD, et al. Cardio-respiratory responses to visually-induced virtual tilts. To be presented at Programming Autonomic Function for Action; Opio Provence, France; September 2-4, 1999.

NATIONAL SPACE BIOMEDICAL RESEARCH INSTITUTE

FINAL PROJECT REPORT

TEAM: **HUMAN PERFORMANCE FACTORS, SLEEP, AND CHRONOBIOLOGY TEAM**

PROJECT TITLE: **SUSTAINED PARTIAL SLEEP DEPRIVATION: EFFECTS ON IMMUNE MODULATION AND GROWTH FACTORS**

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EXECUTIVE SUMMARY

The vulnerability to medical emergencies is greatest in space where there are real limits to the availability or effectiveness of ground based assistance. Moreover, astronaut safety and health maintenance will be of increasing importance as we venture out into space for extended periods of time. It is therefore critical to understand the mechanisms of the regulatory physiology of homeostatic systems (sleep, circadian, neuroendocrine, fluid and nutritional balance) and the key roles played in adaptation. This synergy project has combined aims of the "*Human Performance Factors, Sleep and Chronobiology Team*"; the "*Immunology, Infection and Hematology Team*"; and the "*Muscle Alterations and Atrophy Team*", to broadly address the effects of long term sleep reduction, as is frequently encountered in space exploration, on neuroendocrine, neuroimmune and circulating growth factors. Astronaut sleep is frequently curtailed to averages of between 4-6.5 hours per night. There is evidence that this amount of sleep is inadequate for maintaining optimal daytime functioning. However, there is a lack of information concerning the effects of chronic sleep restriction, or reduction, on regulatory physiology in general, and there have been no controlled studies of the cumulative effects of chronic sleep reduction on neuroendocrine and neuroimmune parameters.

This synergy project represents a pilot study designed to characterize the effects of chronic partial sleep deprivation (PSD) on neuroendocrine, neuroimmune and growth factors. This project draws its subjects from two (of 18) conditions of the larger NSBRI project, "*Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight" (PI:David Dinges)*, one of the projects on the "*Human Performance Factors, Sleep and Chronobiology Team*". For the purposes of this study, to investigate the effects of chronic sleep loss on neuroendocrine and neuroimmune function, we have focused on the two extreme sleep conditions from this larger study: a 4.2 hour per night condition, and a 8.2 hour per night condition.

During space flight, muscle mass and bone density are reduced, apparently due to loss of GH and IGF-I, associated with microgravity. Since >70% of growth hormone (GH) is secreted at night in normal adults, we hypothesized that the chronic sleep restriction to 4 hours per night would reduce GH levels as measured in the periphery. In this synergy project, in collaboration with the "*Muscle Alterations and Atrophy Team*", we have measured insulin-like growth factor-I (IGF-I) in peripheral circulation to test the prediction that it will be reduced by chronic sleep restriction.

In addition to stress, recent research suggests that sleep is also involved in modulation of immune function. While we all have the common experience of being sleepy when suffering from infection, and being susceptible to infection when not getting enough sleep, the mechanisms involved in this process are not understood and until recently have gone largely overlooked. We believe that the immune function changes seen in spaceflight may also be related to the cumulative effects of sleep loss. Moreover, in space flight, the possibility of compromised immune function or of the reactivation of latent viruses are serious potential hazards for the success of long term missions. Confined living conditions, reduced sleep, altered diet and stress are all factors that may compromise immune function, thereby increasing the risks of developing and transmitting disease. Medical complications, which would not pose serious problems on earth, may be disastrous if they emerged in space. Understanding the long-term consequences of sleep curtailment on general health and physiological functioning is critical to the success of any space mission where astronauts will be away from critical care facilities for extended periods of time.

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I. PROJECT RESEARCH ACTVITY

A. HYPOTHESES, OBJECTIVES & SPECIFIC AIMS

The underlying hypothesis of this synergy project was that the reduction of sleep to 50% of habitual sleep duration would lead to decreased growth factor production, and compromised immune function. An underlying debate exists in the sleep and human neurobehavioral literature, such that, on the one hand, it is thought that we can adapt to substantial reductions in nocturnal sleep duration; and on the other, that sleep loss produces cumulative neurobehavioral deficits. Recent evidence from the University of Pennsylvania lab suggests that, at least within a 2-week window, the effects of sustained partial sleep deprivation are cumulative. We had hypothesized that this change would be a consequence of the sleep loss itself, and not simply secondary to stress. This protocol represents an early investigation of this further hypothesis, that partial sleep deprivation leads not only to a cumulative deficit in neurobehavioral output, but also has neuroimmune and neuroendocrine consequences, independent of obvious stress response indicators such as increased cortisol levels. Extensive cytokine testing was performed. There is strong evidence that TNF-alpha and its receptor type p55 are important for immune-related sleep enhancements, and are increased in humans at night (Gudewill et. al., 1992, Mullington et al., 1997). We have measured TNF-alpha, IL-2, IL-1 receptor antagonist. In addition, we have assayed TNF receptors p55 and p75, and IL-10 and high sensitivity C-reactive protein. We hypothesize that sleep is a counter-inflammatory state, and that this has wide ranging implications for health maintenance, immune and metabolic regulation.

The aim of this synergy project was to pilot the investigation of immune and growth factor functioning under conditions of sustained partial sleep deprivation (PSD) where sleep was restricted to approximately 4 hours per night for 10 days. As such, it was not to be a definitive test of our hypotheses, but was intended to enable us to gather pilot data to be used for refining design and hypotheses and to help determine necessary sample sizes for a full study. In order to achieve this aim, we analyzed plasma from subjects in the 8.2 hour control, and in the 4.2 hour sustained PSD conditions of the umbrella-project, "*Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight*" (Human Performance Factors, Sleep and Chronobiology Team). The predictions listed below were developed based on our specific aims.

1. GH secretion is largely suppressed during acute total sleep deprivation, and we anticipated reduced GH release and circulating IGF-I levels as a consequence of sustained PSD.
2. WBCs are increased during acute total sleep deprivation, and we expected to find similar effects due to sustained PSD.
3. It was anticipated that along with the increased levels of circulating WBCs, that we would find an increase in inflammatory cytokines. We also expected to see a decrease in IL-2, as isolated T-cells from subjects who underwent short term PSD showed reduced IL-2 production in response to stimulation (Born et al., 1997).
4. It was anticipated that by the 10^{th} night of PSD, we would see an increase in latent viruses, as measured in urine and blood.
5. Finally, we predicted that obvious signs of stress, such as an elevation of cortisol, would be absent.

B. SUMMARY OF RESEARCH PROJECT

i. PROTOCOL AND METHODS

The design has consisted simply of two conditions extracted from the previously mentioned University of Pennsylvania umbrella study, entitled, "*Countermeasures to Neurobehavioral Deficits from Cumulative Partial Sleep Deprivation During Space Flight*" (David Dinges, PI). The design employed in that study stems from an engineering test method, known as "response surface modeling". This methodology is more completely elaborated in the Dinges group annual report, but simply stated the advantage of this methodology is that it permits the titration of two dependent variables (in this case nocturnal sleep duration and nap sleep duration); and the independent variable of interest. The many small cells of reaction time lapse duration or growth hormone area under the curve permit the extrapolation to predict how these measures will behave under a non-tested combination of nocturnal and nap sleep duration.

The study design involves ninety healthy adults (men and women) who participate in a 14-day ground-based laboratory protocol involving a quasi-random assignment to one of 18 sleep-ration conditions shown in Table 1. ("Quasi-" because we had to run these two conditions this year in order to gather these synergy data.) In the design, nocturnal sleep is centered on the mid-sleep time of the longest (control) sleep duration of 8.2 hours per night (2 am). Nap sleeps are centered 180° out-of-phase with nocturnal mid-sleep time. Extracted for the purposes of this synergy research project were the 8.2 and 4.2 hour per night conditions, indicated in bold in Table 1. Because these represent only two of the 18 conditions in the study, we could not apply the response surface methodology, but have analyzed these data as a 2x2 design (2 conditions (8.2 vs. 4.2); by 2 times (baseline and 10th PSD day)). We have characterized the effects of 50% reduction in sleep time on growth factors, neuroendocrine and neuroimmune parameters, as a preliminary investigation into our hypothesis that partial sleep deprivation leads not only to a cumulative deficit in neurobehavioral output, but also to altered neuroimmune and neuroendocrine function, specifically, reduced IGF-I levels and increased inflammatory cytokines.

Table 1. Conditions Summary for Response Surface Experimental Design

Total time in bed for each of 18 conditions				
Duration of nap sleep opportunity	Duration of nocturnal sleep opportunity			
	8.2	6.2	5.2	4.2
0	**8.2**	6.2	5.2	**4.2**
.4	---	6.6	5.6	4.6
.8	---	7.0	6.0	5.0
1.2	---	7.4	6.4	5.4
1.6	---	---	6.8	5.8
2.0	---	---	7.2	6.2
2.4	---	---	---	6.6

Subjects

Inclusion/exclusion criteria include:
1) age 25 - 50 yrs

2) body mass index within 10% of normal
3) no shift work or irregular sleep/wake routine in past 60 days
4) sleep log evidence of a stable sleep-wake cycle as defined by habitual nocturnal sleep duration of 6.5 - 8.5 hr, habitual morning awakening between 0600 and 0900 hr, and no evidence of habitual napping (i.e., > 1/wk)
5) no sleep disorder (determined by history, actigraph, baseline PSG)
6) no history of depression, mania, psychosis, epilepsy, or thyroid disorders
7) no evidence of drug use by urinary toxicology screens
8) no indication of infection or disease by blood chemistry tests
9) Medical exam, history, blood and urine testing all reveal no chronic or debilitating medical conditions

Subjects were 4 women and 6 men, between the ages of 26-38 (average 31.1yrs). Conditions were gender-balanced, three men and 2 women in each. There were repeated problems with the catheter in one of the women in the PSD condition, and it finally had to be removed. Blood for this subject's WBC and PCR data (latent viruses measured in blood samples) were collected by butterfly needle and vacutainer.

Subjects spent 14 days (24-hr periods) in the time-isolated, temperature controlled University of Pennsylvania Sleep and Chronobiology Laboratory in no more than 50 Lux of ambient light (mean 41 Lux). Subjects for this synergy project were scheduled to have 8.2 hr of available sleep time, or time in bed, for the first two nights in the protocol. For the next 10 nights in the study they had either 4.2 hours of available sleep time per night, or if they were in the control group, continued with 8.2 hours per night. On the last night in the study, all subjects were permitted 14-hrs of recovery sleep. Throughout all wake periods subjects were monitored to ensure that they remained awake. Ambulatory EEG/EOG, and core body temperature were recorded throughout each waking day. Outside of blood collection days, throughout scheduled wake periods, subjects were tested at regular intervals on a computerized neurobehavioral test battery. Nutritionally balanced meals were provided throughout the protocol, at appropriate times for breakfast, lunch, and dinner (caffeine and alcohol were prohibited). Meals were prepared in the General Clinical Research Center metabolic kitchen for the subjects, moved to the laboratory kitchen, and served at regular meal times. Subjects were not permitted to have visitors during the protocol. Specific neurobehavioral and electrophysiological methods are described in the annual report for the umbrella project.

Specimen Sampling and Processing

On the first day and night, and on the 10th day and night of the experimental phase of the protocol, subjects wore an indwelling catheter, and blood was drawn at 15-minute intervals. Catheters were inserted by a nurse, at approximately 14:30, and blood was drawn, starting at 16:30 and continuing until 17:30 the following evening. The delay in blood collection after insertion of the catheter is to prevent contamination of data by a stress induced surge in hypothalamic-pituitary-axis hormones. A blood pump (Carmeda ConFlo™) was used, together with a heparin-lined catheter system, and 2ml samples were pumped into a vacutainer over 15 minutes. We successfully collected 100 integrated blood samples, into EDTA prepared glass vacutainers, on each of the two 25 hour sampling days for each subject for a total of >1800

samples (repeated problems with the catheter of one subject in the 4.2 hour nocturnal sleep condition led to excluding her from serial blood sample analyses). On blood collection days, subjects remained in bed for the collection period, and were only permitted out of bed for bathroom breaks. They did not undergo neurobehavioral testing on these days.

Vacutainer tubes with EDTA were set on ice during the 2ml collection over 15 minutes. Once the blood was collected, it was taken and centrifuged immediately at 2600xg for 7 minutes at 4°C. Following centrifugation, plasma pipetted into polypropylene tubes and frozen to –70°C for later assay. These samples were used for the analysis of cortisol (Diagnostic systems Laboratories, Webster, TX), melatonin (IBL, Hamburg, Germany), GH (Nichols International, Inc.), and IGF-I (Diagnostic systems laboratories, Inc., Webster, TX) using RIA techniques. Intra assay coefficients of variation were as follows: for cortisol, 5.5%, GH 6.4%, melatonin 12.5 for values under 10pg/ml; 8.3% for values >10pg/ml), and for IGF-I was <4%.

TNF-alpha, TNF receptors p55 and p75 (all by Biosource, Fleurus, Belgium), IL-1 receptor antagonist (R&D Systems, Minneapolis, MN), IL-2 (R&D Systems, Minneapolis, MN) and IL-10 (OPTEIA, San Diego, CA), were all assayed using ELISA techniques. The intra assay coefficient of variation was <5% for IL-2, <6.5% for TNF-alpha and receptors, 12% for IL-10, and for IL-1 receptor antagonist was 15.5%. C-reactive protein was measured using a high sensitivity latex-enhanced immunoassay (Dode Behrings, Newark,DE).

Extra samples were collected at 09:00h, into EDTA vacutainers for WBC and differential counts. In order to collect these blood samples, the pump was paused between samples and a vacutainer was used to draw the sample through the catheter. The sample was then sent directly to the GCRC laboratory for processing.

At 07:00h on both days in each condition, 15 ml sample was drawn into acid citrate dextrose (ACD) and sent via same-day FedEx to our collaborators on the immunology team: Janet Butel, Ph.D., John Lednicky, Ph.D., and Paul Ling, Ph.D, at Baylor College of Medicine, for assessment of latent viruses (Epstein-Barr virus, BK and JC viruses, and SV40) using quantitative PCR techniques. In addition to blood samples, urine was also collected for PCR analysis. A mid stream sample was collected from the first void of each collection day, and frozen to -70°C, stored for later analysis. On this morning of the protocol, subjects also gave a urine sample that was frozen and later sent to the Baylor lab, also for assessment of latent viruses using PRC analysis techniques. Colleagues at Baylor processed the samples as soon as they received them. PBMC were selectively collected after differential centrifugation through a ficoll-hypaque gradiant (Accuspin system-Histopaque-1077 centrifuge tube). The PBMC were collected, washed once in PBS, aliquoted to approx. 1 X 10^7 cells/cryovial, re-centrifuged to pellet the cells, and then stored at –70 °C. Total DNA was extracted from the PBMC pellets using a QIAGEN QIAamp blood kit.

Oligonucleotides and PCR Analysis

PCR and DNA sequencing oligonucleotide primers that were used in this study were purchased from GIBCO-BRL or from Integrated DNA Tech. Primers used for analysis of the SV40 regulatory region (primer pair RA1 and RA2) were described previously (Lednicky et al., 1995). Plasmids pSVSph21-N and pSV21-N were used as positive control templates for PCR analysis of SV40 and were described previously (Lednicky and Butel, 1997). PCR amplification and DNA sequence analysis of the BKV and JCV regulatory regions were performed with primer pairs BK1, BK2, and JC1, JC2, respectively (Markowitz et al., 1991). Plasmid pBK-

Dunlop (obtained from the American Type Culture Collection) and pJC-MAD-1 (a gift to the Butel laboratory from M. Sullivan) were used as positive control templates for PCR analysis of BKV and JCV, respectively.

PCR amplifications were performed in a Perkin Elmer GeneAmp PCR System 2400 thermocycler for a total of 45 denaturation, annealing, and extension steps using the annealing temperatures specific for each primer pair as described (Lednicky and Butel, 1997). Samples were first denatured at 94°/2 min, followed by 44 cycles of denaturation for 94°C/15 sec, annealing at 60°C or 63°C/15 sec, and extension at 72°C/15 sec, followed by a terminal cycle of 94°C/15 sec, 60°C or 63°C/15 sec, and 72°C/7 min. The samples were then held at 4°C until processing time. Each PCR reaction contained either 500 ng of PBMC DNA (approx. 8 X 10^4 cell equivalents) in a 50 µl reaction volume or DNA extracted from urine pellet (obtained from 1 ml of urine) in a 100 µl reaction. PCR reactions contained commercially prepared buffer with magnesium ions (Perkin-Elmer Roche), each dNTP at a concentration of 200 µM, and either 5 units of TAQ polymerase (AmpliTAQ, Perkin Elmer Roche) for a 50 µl reaction volume (PBMC analysis) or 10 units TAQ for a 100 µl reaction volume (urine analysis).

PCR reactions were analyzed on a 2% ethidium-bromide-stained agarose gel that was viewed under UV light.

ii. RESULTS

Inflammatory Markers, Including Cytokine Measurements

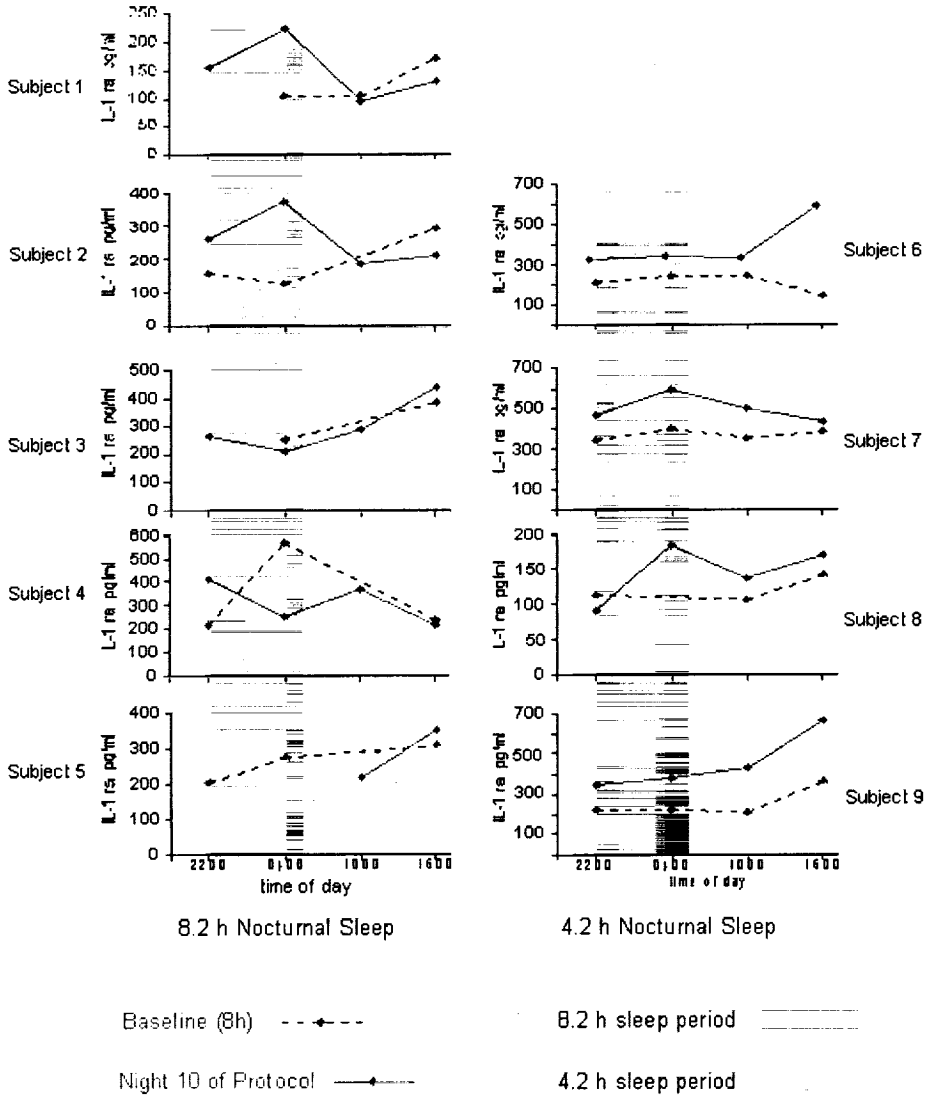
Changes in white blood cells were consistent with patterns seen in response to total sleep deprivation. Lymphocyte and monocyte numbers rose from baseline through to after the 10^{th} night of PSD. While these changes were small, they were evident in 4 of 5 subjects. Table 2 summarizes the data and provides the results of paired t-testing, showing trends ($p<0.10$) towards an increase in lymphocyte and monocyte number, as well as a significant increase in platelets. All subjects had increased platelet numbers after 10 nights of PSD.

Cytokines involved in the regulation of inflammatory processes were measured, including TNF-alpha, TNF-receptors p55 and p75, IL-1 receptor antagonist and IL-10. IL-2, a major T-cell regulating cytokine, previously shown to be altered by sleep loss, was also measured. While there was no effect of partial sleep deprivation on the TNF family of cytokines, IL-1 receptor antagonist was significantly elevated ($p<0.05$) in the PSD group, as was high-sensitivity C-reactive protein ($p<0.05$). We consider this to be of significant importance considering the predictive value of high-sensitivity C-reactive protein levels in cardiovascular risk (Ridker et al., 1998). The left panel of Figure 1 shows each of the subject IL-1ra profiles under a normal sleep-wake schedule, and on the right, the subjects who had undergone sleep reduction to 4 hours per night for 10 nights. The recently submitted abstract (Meier-Ewert et al., 2000, submitted for presentation at the "Bioastronautics Investigators' Workshop, Jan 17-19, 2001") provides a synopsis of high sensitivity C-reactive protein findings, data will be presented at the meeting.

Table 2. Blood test results for subjects in the sustained partial sleep deprivation (PSD) condition.

Blood Parameter	Baseline	Partial Sleep Dep.	p-value
Total red blood cells (THO/μL)	4.2 (0.5)	4.4 (0.6)	P=0.37
Total white blood cells (MIL/μL)	5.0 (0.7)	6.2 (1.6)	P=0.10
Granulocyte # /μL	3296 (421)	4040 (1566)	P=0.28
Granulocyte %	65.4 (5.6)	63.6 (9.4)	P=0.62
Lymphocyte # /μL	1340 (320.9)	1560 (230.2)*	P=0.09
Lymphocyte %	26.8 (5.5)	27.2 (8.7)	P=0.90
Monocytes # /μL	304 (95.3)	420 (192.3)*	P=0.09
Monocyte %	5.8 (1.3)	6.8 (2.3)	P=0.18
Platelet count (THO/μL)	217.4 (32.8)	241.0 (29.2)*	P=0.003
Hemoglobin (g/dL)	13.2 (1.6)	13.0 (2.1)	P=0.62
Hematocrit (%)	38.2 (3.4)	37.0 (4.5)	P=0.14

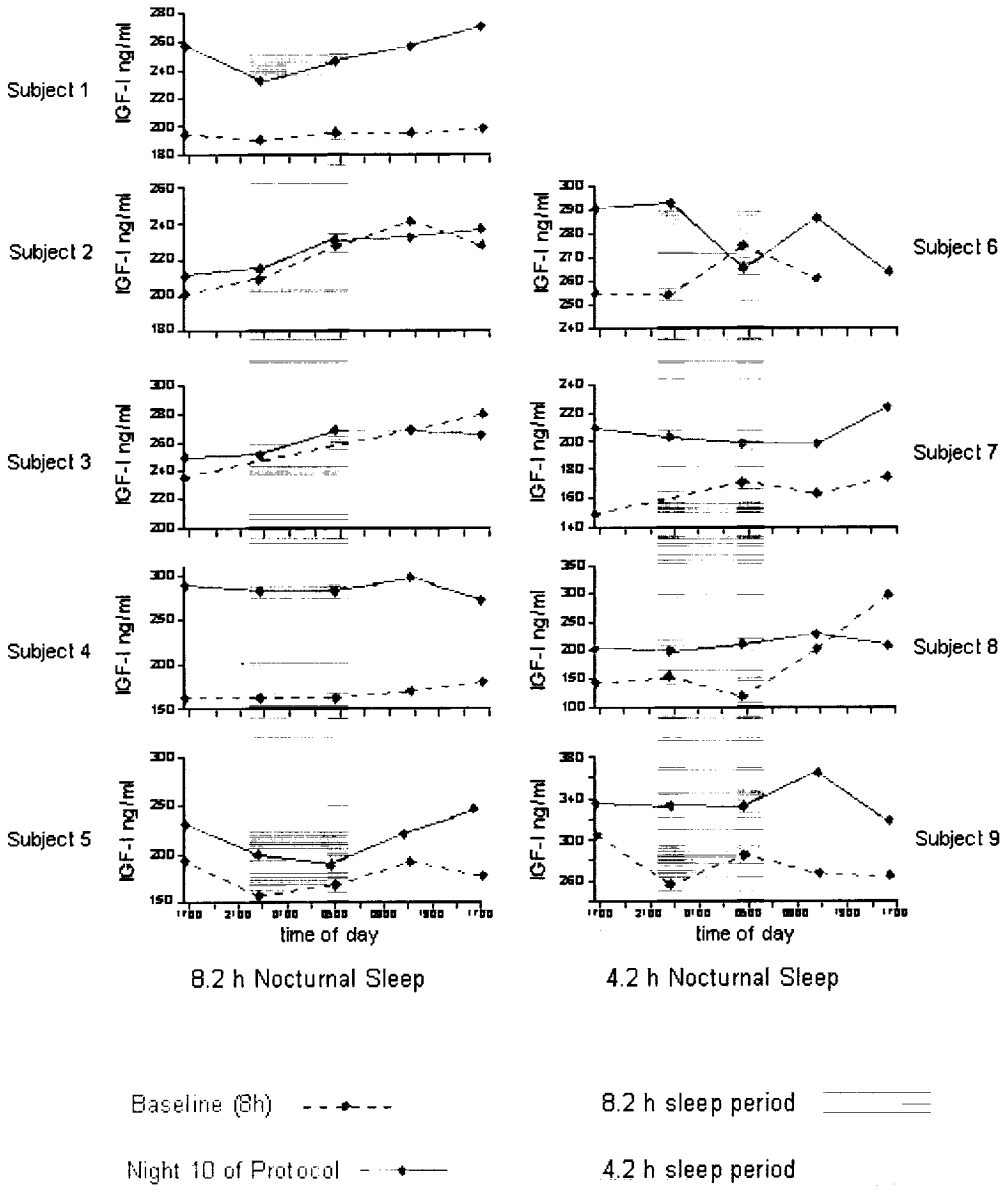
Figure 1. IL-1 receptor antagonist levels for individual subjects at baseline and after 10 days in the protocol with nocturnal sleep opportunity of either 8.2 or 4.2 hours/night.



Growth Factors

In addition to cortisol, melatonin and growth hormone, measured in the larger study, we measured IGF-I. As can be seen in Figure 2, we found an increase in this growth factor during sustained PSD. While there appears to be a noteworthy increase in IGF-I in 2 of the 5 subjects who were in the control group, all 4 of the subjects who were in the PSD condition showed increased IGF-I levels. Paired t-tests found significant differences ($p<0.02$) in the daily average value of IGF-I after 10 nights in the PSD condition, but not in the control condition.

Figure 2. IGF-I levels for individual subjects at baseline and after 10 days in the protocol with nocturnal sleep opportunity of either 8.2 or 4.2 hours/night.



Latent Viruses

PBMC DNA extracted from blood collected from the PSD subjects were tested for the presence of BKV, JCV, and SV40. Analyses for EBV in blood were negative. PBMC DNA from each subject at both collection times were tested. PCR was first attempted with primers AG1 and AG2 to test whether the samples could be used for PCR reactions. All samples appeared suitable for PCR analysis, since specific PCR products were formed. All samples were negative for BKV, JCV, and SV40 DNA sequences.

DNA extracted from sedimented urine pellets derived from urine collected from subjects were tested for the presence of BKV, JCV, and SV40. DNA from each subject at both collection times was tested. PCR was first attempted with primers AG1 and AG2 to test whether the samples could be used for PCR reactions. Only urine from one subject (in the PSD condition), subject # 7, was positive for JCV (at both baseline and following 10 nights of PSD). No SV40 or BKV was detected in any urine samples.

We concluded that under the conditions of partial sleep deprivation tested in this study, polyomaviruses and EBV were not reactivated.

Iii DISCUSSION AND CONCLUSIONS

The increase in the WBCs was expected based on a dearth of reports in the literature showing an increase in neutrophils in response to total sleep deprivation (Herz 1923; Kuhn et al., 1969; Dinges et al., 1994), as well as lymphocytes and monocytes (Dinges et al., 1994; Born et. al., 1997). In addition, one previous report found that a single night of partial sleep deprivation led to increased lymphocyte numbers (Irwin et al., 1996). Our finding of increased monocytes is consistent with the increased IL-1 receptor antagonist and high sensitive C-reactive protein levels following partial sleep deprivation. Furthermore, the elevation in platelets is also consistent with increased inflammatory factors.

These changes in immune parameters, induced by chronic partial sleep deprivation, are intriguing. They suggest that the change in immune function is stable, and implicates the blood cells involved in inflammatory processes. However, it is also noteworthy that the other major players in the pro-inflammatory immune system are not elevated, specifically, TNF-alpha and its receptors p55 and p75. IL-1 cannot be reliably measured in human plasma, but it is highly correlated with IL-1 receptor antagonist. Therefore, it is likely that IL-1 is also elevated due to chronic reduction in nocturnal sleep.

TNF-alpha receptor p55, rather than p75, has been specially implicated in the sleep enhancement seen to follow endotoxin challenge in animal models (Fang et al., 1997, Lancel et al., 1997). However, it is clear that there are multiple immune-pathways that are capable of increasing sleep. While control mice show an increase in amount of time spent in non-REM sleep following a TNF challenge (intraperitoneal administration), TNF-receptor p55 knockout mice failed to show this response (Fang et al., 1997). However, intraperitoneal injections of IL-1-beta to TNF receptor p55 knockout mice did, nonetheless, show increased non-REM sleep duration, demonstrating that the routes by which these different cytokines induce their common

sleep effects are multiple. Based on these findings we had expected TNF-alpha and receptor p55 to increase through the sleep reduction period, as a consequence of increased sleep pressure. The failure to find an increase in this cytokine receptor may be due to the small number of subjects in the study or may imply that the sleep deficit accumulated in this study is not great enough to effect the TNF-system.

The IGF-I results were unexpected, and may be associated with changes in growth hormone (results of GH and cortisol analyses from these subjects are being reported by Dinges et al., in the annual report "*Countermeasures to neurobehavioral deficits from cumulative partial sleep deprivation during space flight*". They show a shifting and slight reduction of GH in the nocturnal period, and a slight elevation in the nadir of cortisol). The IGF-I data shown in figure 1 are within normal range for healthy adults between 25-40 years of age. IGF-I is important for the maintenance of muscle and bone systems and there is evidence that the GHRH-GH-IGF-I axis is disrupted during space flight (Hymer et al., 1985; Grindeland et al., 1987; Roy et al., 1996). Despite remaining within the normal range, the data shows that every subject in the PSD condition had increased IGF-I levels. In the control group, 2 out of 5 subjects also showed this pattern, while a third subject had a slight elevation over baseline. It is known that conditions of high stress (Opstad and Aakvaag, 1983), malnutrition, and being elderly and frail (Rosen and Conover, 1997) are all conditions that **reduce** IGF-I levels. We had expected that prolonged reduction of nocturnal sleep to 50% of its normal duration would lead to decreased levels related to anticipated GH reductions. Instead, we found increased levels of IGF-I. Since these are young volunteers, carefully selected for being in good health, it is unlikely that the increase in IGF-I is associated with inadequate nutrition before coming into the study. (None the less, we will carefully monitor diet before and during future protocols.) While it is true that subjects in the Sleep and Chronobiology Laboratory have restricted movement (they can't engage in heavy exercise while in the study), previous studies have failed to find an effect of transient strenuous exercise on IGF-I levels, even though GH levels do increase with exercise training (Kraemer et al., 1995). It is therefore unlikely that the increased IGF-I levels we see here are due to stress.

Increased levels of IGF-I have been associated with higher delta wave sleep in healthy elderly (Prinz et al., 1995) and it may be that the increased levels of IGF-I are associated with increased sleep pressure. There are no available IGF-I data collected from controlled acute sleep deprivation studies. Therefore, we will analyze IGF-I from plasma collected before and after 88 hours of total sleep deprivation (a separate ongoing study, Dinges, PI), in order to determine whether these results are associated with homeostatic sleep pressure. While the cause is not clear, the finding of increased IGF-I warrants further investigation, particularly in light of recent evidence that implicates increased IGF-I levels in various forms of cancer (Yu et al., 1999; Petridou et al., 1999). Sleep loss may have metabolic consequences that, in combination with long term space travel, could be potentially hazardous. We have recently assayed samples from this study for leptin levels, and find them to be decreased during prolonged partial sleep deprivation (Mullington et al., 2000), further indication that neuroendocrine and metabolic indices are altered by a sleep deficit.

Based on these preliminary findings, we conclude that there is a need for furthur rigorous investigation into the hypothesis that sleep is a counter-inflammatory state and that sleep loss alters the homeostasis of metabolic, immune and inflammatory host-protective functions.

II. IMPLICATIONS FOR FUTURE RESEARCH & PLANS

We are planning to continue collaborations with Janet Butel and colleagues at Baylor College, measuring latent viruses in urine samples taken from subjects undergoing varying degrees of sleep loss in our NIH funded study of the effects of chronic sleep restriction on human host response. The number of subjects in this study was small, and since reactivation of latent viruses is more apparent in older subjects, it will be necessary to continue to assess specimens from additional subjects, stratified by age. In subjects under 35 years of age, shedding of JC virus in urine is quite rare, but becomes less infrequent in subjects over 35. Of the 10 subjects investigated in this study, only 3 were 35 or over.

We are currently conducting further studies to investigate the effects of partial sleep deprivation on human host response. The PI has recently been awarded an R01 application to the NIH to investigate host response to *E-coli* endotoxin following 10 nights of sleep restricted to 4 hours per night. This will enable simultaneous investigation of the *in vivo* response to endotoxin and the *in vitro* response of stimulated cells taken just prior to *in vivo* challenge. In addition, this will allow the characterization of basal changes in neuroendocrine and neuroimmune parameters. The interesting and unexpected findings of increased IGF-I levels will be further characterized under conditions of sleep loss in our ongoing NIH study.

The results of this pilot study have shown that chronic sleep restriction has neuroendocrine and immunomodulatory effects that are important for maintaining the health of astronauts during extended space travel. New areas for further research have been identified that will help determine the role of sleep in neuroendocrine and neuroimmune regulation. The findings of this study will therefore be important for the development of health maintenance and illness prevention strategies in space as well as on earth.

The C-reactive protein and IL-1 receptor antagonist increase, coupled with the increased platelets and white blood cell counts provide clear support for the hypothesis that sleep is a counter-inflammatory state and further implicate sleep in immune homeostasis. There is a need for further investigation into the hypothesis that sleep loss alters the homeostasis of metabolic, immune and inflammatory host-protective functions.

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APPENDIX A. Publications supported through NSBRI funding

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APPENDIX B. One copy of papers published or submitted and supported by NSBRI (for original signed submission only).